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Aspergillus fumigatus and Aspergillosis

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INTRODUCTION

Aspergillus fumigatus is a saprophytic fungus that plays an essential role in recycling environmental carbon and nitrogen (235, 506, 676). Its natural ecological niche is the soil, wherein it survives and grows on organic debris. Although this species is not the most prevalent fungus in the world, it is one of the most ubiquitous of those with airborne conidia (443, 444, 466). It sporulates abundantly, with every conidial head producing thousands of conidia. The conidia released into the atmosphere have a diameter small enough (2 to 3 μm) to reach the lung alveoli (518, 577). *A. fumigatus* does not have an elaborate mechanism for releasing its conidia into the air; dissemination simply relies on disturbances of the environment and strong air

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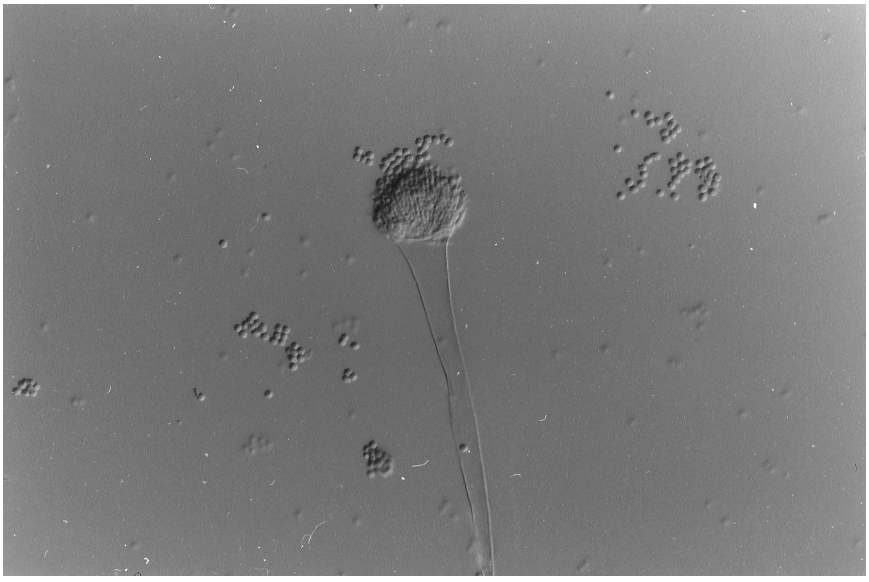


FIG. 1. Light microscopy of typical *A. fumigatus* sporulating structures.

currents. Once the conidia are in the air, their small size makes them buoyant, tending to keep them airborne both indoors and outdoors. Environmental surveys indicate that all humans will inhale at least several hundred *A. fumigatus* conidia per day (99, 222, 271). For most patients, therefore, disease occurs predominantly in the lungs, although dissemination to virtually any organ occurs in the most severely predisposed.

Inhalation of conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated relatively efficiently by innate immune mechanisms. Thus, until recent years, *A. fumigatus* was viewed as a weak pathogen responsible for allergic forms of the disease, such as farmer's lung, a clinical condition observed among individuals exposed repeatedly to conidia, or aspergilloma, an overgrowth of the fungus on the surface of preexisting cavities in the lungs of patients treated successfully for tuberculosis (169, 341, 500). Because of the increase in the number of immunosuppressed patients, however, and the degree of severity of modern immunosuppressive therapies, the situation has changed dramatically in recent years (114, 556, 572). Over the past 10 years, *A. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts in developed countries (13, 43, 61, 142, 170, 231). A fourfold increase in invasive aspergillosis (IA) has been observed in the last 12 years. In 1992, IA was responsible for approximately 30% of fungal infections in patients dying of cancer, and it is estimated that IA occurs in 10 to 25% of all leukemia patients, in whom the mortality rate is 80 to 90%, even when treated (59, 140, 141, 231, 682). IA is now a major cause of death at leukemia treatment centers and bone marrow transplantation (BMT) and solid-organ transplantation units (119, 159, 489, 575).

Although *A. fumigatus* is the most common etiologic agent, being responsible for approximately 90% of human infections (61, 159, 169, 334, 350, 587, 676), it is not the only pathogen in this genus. *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* can also cause human infections. Since *A. fumigatus* is the most common, however, this review is devoted exclusively to it. Fundamental and clinical aspects of the pathobiology of *A. fumigatus* infections are presented, with special emphasis on IA. The topics discussed include (i) taxonomic characterization of the species, (ii) clinical and laboratory diagnosis of the disease, (iii) host immune response to the fungus and putative fungal virulence factors, and (iv) antifungal drugs used in treatment.

TAXONOMY OF *A. FUMIGATUS*

Species Identification

Culture and morphological characteristics. Identification of *A. fumigatus* is based predominantly upon the morphology of the conidia and conidiophores. The organism is characterized by green echinulate conidia, 2.5 to 3 μm in diameter, produced in chains basipetally from greenish phialides, 6 to 8 by 2 to 3 μm in size. A few isolates of *A. fumigatus* are pigmentless and produce white conidia (582). The chains of conidia are borne directly on broadly clavate vesicles (20 to 30 μm in diameter) in the absence of metulae (Fig. 1). No sexual stage is known for this species. *A. fumigatus* is a fast grower; the colony size can reach 4 ± 1 cm within a week when grown on Czapek-Dox agar at 25°C (518). *A. fumigatus* is a thermophilic species, with growth occurring at temperatures as high as 55°C and survival maintained at temperatures up to 70°C (235, 341, 518, 577).

A. fumigatus is morphologically more variable (361, 576, 595) than was originally described by Raper and Fennell (518). These variations have led to the description of several varieties of *A. fumigatus*, including *acolumnaris*, *phialiseptus*, *ellipticus*, and *sclerotiorum*, with the distinctions being based on only slight morphological differences. *A. fumigatus*, *A. brevipes*, *A. duricaulis*, *A. unilateralis*, *A. viridinutans*, together with anamorphs of species within the perfect genus *Neosartorya*, a genus in which morphologically related species have been grouped, are classified as *Aspergillus* sect. *fumigati*. The search for a sexual stage of *A. fumigatus* has been attempted among *Neosartorya* species, since it would allow classical genetics to be pursued in *A. fumigatus*. To date, no such stage has been discovered.

Biochemical and molecular characterizations used in species determination. The need for a better taxonomic definition of the species *A. fumigatus* and the possible misidentification of

TABLE 1. Selected features used to classify *A. fumigatus* to the species and strain levels

Feature	Reference(s)
Species characterization[a]	
Culture and morphology	341, 518, 577, 595
Secondary metabolites	200
Specific unique and repeated DNA sequences[b]	105, 211, 218, 502, 582
DNA-DNA reassociation	501
Strain characterization	
Microsatellite patterns	34
Hybridization patterns of endonuclease-digested DNA with *AFUT1*	133, 217

[a] Isoenzyme patterns (369, 400, 543, 554), ethidium-bromide visualized RFLPs (82, 144), and IGS probes (514, 618) can be used to rank strains at a subspecies level.

[b] Used directly for comparative analysis of sequence data obtained with unique nuclear (211) or ITS1 and ITS2 ribosomal (105, 210, 502) genes or indirectly in Southern hybridization experiments (218, 582).

a teleomorph stage of *A. fumigatus* among the *Neosartorya* species have led to the study of selected biochemical and molecular criteria, in addition to morphological data, as adjuncts to species determination. Biochemical characterizations which have been studied include the detection and identification of secondary metabolites (200), the identification the ubiquinone system (400), and the examination of isoenzyme patterns (369, 400, 543, 554). Molecular data have been obtained on total DNA (105, 218, 501), mitochondrial DNA (mtDNA) (127, 543) or ribosomal DNA (rDNA) (105, 127, 210, 543, 618) by using various methodological approaches, mainly restriction fragment length polymorphisms (RFLP) visualized with or without hybridization to specific probes and sequencing of characteristic DNA regions. Criteria which have been suggested as useful in the identification of *A. fumigatus* are summarized in Table 1.

The profiles of secondary metabolites, including mycotoxins and antibiotics, produced by *A. fumigatus* and its morphological variants listed above are similar. Fumagillin, fumitoxin, fumigaclavines, fumigatin, fumitremorgins, gliotoxin, monotrypacidin, tryptoquivaline, helvolic acid, and metabolites of two chromophore families uncharacterized chemically (FUA and FUB) are the secondary metabolites most commonly found in *A. fumigatus* (200). In contrast, anamorphs of *Neosartorya* species, as well as other members of *Aspergillus* sect. *fumigati* (*A. brevipes*, *A. duricaulis*, *A. unilateralis*, and *A. viridinutans*), produce few of these secondary metabolites. The number of isoprene side chains of the ubiquinone molecules, which is 10 in the *Aspergillus* sect. *fumigati*, cannot be used for identification at the species level, since all species of this section, including all *Neosartorya* anamorphs, have the same number of isoprene units (400).

Several investigators have attempted to use the analysis of isoenzyme patterns as a taxonomic tool (369, 400, 543, 554). The only enzyme pattern common to all strains of *A. fumigatus* is glutamate dehydrogenase. Some enzymes (lactate dehydrogenase, superoxide dismutase, isocitrate dehydrogenase, aspartate aminotransferase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase) have been reported to be monomorphic, although data vary from study to study, and other enzymes (malate dehydrogenase, glucose phosphate isomerase, phosphoglucomutase, hexokinase, esterase, malate dehydrogenase, peptidases, fructose kinase, purine nucleoside phosphorylase, and phosphatases) display polymorphic patterns. However, since multilocus enzyme electrophoresis patterns of closely related species have not been investigated, their usefulness as a general taxonomic tool is unknown.

Analytical approaches which involve the analysis of DNA have shown more promise in the characterization of *A. fumigatus*. One useful criteria is DNA-DNA reassociation values, wherein values higher than 92% have been found for strains of *A. fumigatus*, while values lower than 70% have been calculated for *A. fumigatus* and *Neosartorya* species, indicating that the two genera are genetically distinct (501). Another helpful approach to the study of nuclear DNA has been the analysis of introns and the sequencing of entire or significant portions of unique genes such as the β-tubulin and hydrophobin genes (105, 211). A third successful method is the hybridization of endonuclease-digested DNA with various *A. fumigatus*-specific unique or repeated (see below) DNA sequences (218, 582). Amplification by PCR of specific sequences, originally identified by random amplification, seems promising (71). While sequencing of the internally transcribed spacers ITS1 and ITS2 of rDNA has not been completed, there appear to be sufficient differences to distinguish *Neosartorya* species and *A. fumigatus* (105, 210, 502).

Other DNA-based approaches have not been useful in the speciation of *A. fumigatus*. For example, pulse field gel electrophoresis has shown the presence of five chromosomal-sized DNA bands ranging in size from 1.7 to 4.8 Mb (651). It is not known, however, if each of these bands corresponds to one or two chromosomes, as has been shown with *Aspergillus* spp. other than *A. fumigatus* (635). Moreover, comparisons of chromosomal banding patterns of taxonomically related species have not been done. The analyses of mtDNA and rDNA have produced limited results. RFLPs in mDNA were not observed among 60 strains of *A. fumigatus* when DNA was digested with *Hae*III alone or in combination with other enzymes. Moreover, the same pattern was observed with the closely related species, *Neosartorya fischeri fischeri*. An approach which has not been attempted, but which could be helpful, is the use of AT-rich recognition enzymes for digesting mtDNA. The use of such enzymes has proved beneficial in characterizing the *flavi* section of the aspergilli (127, 543). As with other fungi, investigations of the sequences of the 18S and 28S subunits of rDNA have shown that there is insufficient variability for this method to be useful taxonomically. Southern hybridization with intergenic spacer (IGS) probes from non-*fumigatus* species showed that all isolates of *A. fumigatus* tested had common major fragments with a variable number of 200-bp repeat units, suggesting that the IGS region was too heterogeneous to be used at a species level (618).

In summary, secondary metabolites and sequencing data, as well as DNA-DNA reassociation values and Southern hybridizations patterns with single and repeated sequences or PCR amplicons have been useful criteria for the taxonomic characterization of *A. fumigatus* (Table 1). They prove that *A. fumigatus* and *N. fischeri*, whose anamorphic stage is very closely related to *A. fumigatus*, are two separate species genetically and biochemically. Therefore, the search for a teleomorph stage of *A. fumigatus* must continue.

Molecular Analyses in Strain Typing

Aside from its fundamental interest, intraspecific characterization of this species has potent epidemiological and clinical implications. Since strain typing requires methods that are highly discriminative, reproducible, and independent of growth conditions, phenotypic analysis based on protein patterns detected by antibodies or enzymatic substrates should be discour-

aged (84, 637). At best, protein patterns can be used to rank strains at a subspecies level. In contrast, genotypic methods are independent of the external milieu. Some of the earlier molecular methods, however, are helpful only for analysis at the subspecies level. RFLP following digestion of total genomic DNA by *Xba*I, *Sal*I, or *Xho*I shows a limited degree of discrimination among strains. The complex banding patterns with large numbers of faint bands displayed in ethidium bromide-stained gels are difficult to interpret, and only major bands can be used to designate subspecific clusters (82, 144). Similarly, heterogeneity in the IGS region can be used to group strains of *A. fumigatus* only at a subspecific level (514, 618).

Only three methods can be used to genotypically type *A. fumigatus* strains. Two of these methods use PCR, each with different primers and amplification protocols (microsatellite and random amplified polymorphic DNAs [RAPD]), whereas the third uses RFLP visualized after hybridization with a repeated DNA sequence.

RAPD is the method most commonly used to type strains of *A. fumigatus* (11, 23, 81, 354, 369, 385, 422, 670). To date, the decamer primer R108 (GTATTGCCCT) generated the best strain differentiation (23). However, RAPD patterns are difficult to repeat or interpret due to the low annealing temperature (44, 386, 687). Moreover, the distance of migration scanned is only a few centimeters, and the variability in banding pattern is too limited to make the comparison of a large number of strains feasible. The second PCR-based method involves microsatellites. This method, which has been used to construct the physical map of the human genome, has been successfully applied recently to *A. fumigatus* (34). The method is rapid and highly reproducible and, in contrast to RAPD, uses unique primers and specific sequences flanking the microsatellite. Four CA repeats have been identified to date: $(CA)_9(GA)_{25}$, $(CA)_2C(CA)_{23}$, $(CA)_8$, and $(CA)_{21}$.

Hybridization of restriction enzyme fragments with repeated DNA sequences, a method successfully used to type other fungal pathogens, has also been used to type *A. fumigatus* strains. Screening of a phage library resulted in the isolation of a phage (λ3.9) which contains a species-specific repeat sequence. Use of this phage as a probe provides unique and highly discriminative Southern blot hybridization patterns for each strain tested (133, 217). The repeat sequence *AFUT*1, inserted into phage λ3.9 and used for strain fingerprinting, is a defective retrotransposon element of 6.9 kb bounded by two long terminal repeats (LTR) of 282 bp (459) (Fig. 2). The 5′ and 3′ LTRs are not totally homologous, since they have only 90% identity. Moreover, the 5′ LTR of another copy of *AFUT*1, isolated from a different phage (λ4.11), which cross-hybridizes with λ3.9, is 86.5% identical to the 5′LTR of the retrotransposon isolated from the λ3.9 phage. A 5-bp duplication site was found at the border of *AFUT*1. *AFUT*1 encodes amino acid sequences homologous to the reverse transcriptase, RNase H, and endonuclease encoded by the *pol* gene of retroviruses.

Comparison of *AFUT*1 with other fungal and nonfungal LTR retrotransposons showed that *AFUT*1 has a sequence and organization characteristic of the gypsy family of *Drosophila* (459). At least 10 copies of the retrotransposon element are found in the genome of *A. fumigatus*. However, *AFUT*1 is a defective element; the putative coding domains contain multiple stop codons due exclusively to transitions from C · G to T · A. Such a pattern of nucleotide variation is reminiscent of the repeated-induced point mutation (RIP) in *Neurospora* repeated sequences. However, no sexual reproduction is known in *A. fumigatus*, and no methylation of cytosine, an event typically associated with mutations in sequences affected by RIP,

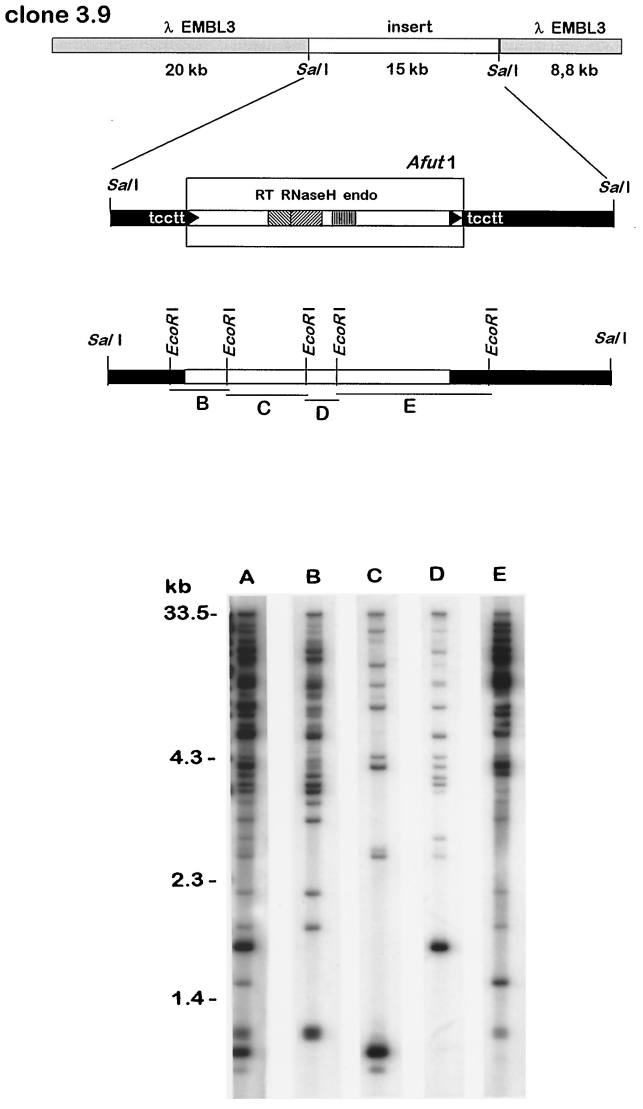


FIG. 2. Schematic representation of the λ3.9 probe of *A. fumigatus* used for molecular studies and typical hybridization patterns obtained with *Eco*RI-digested total DNA probed with the entire *Sal*I-*Sal*I fragment (A) and *Eco*RI fragments of the repeated sequence (B to E). The repeated element *Afut*1 (squares) is an inactive retroelement of 6.9 kb bounded by two LTRs (▶) and with sequences homologous to reverse transcriptase (RT), RNase H, and endonuclease (endo) encoded by the *pol* genes of retrotransposons.

was detected (459). This result would suggest that *AFUT*1 was subjected to RIP at a time when *A. fumigatus* possessed a functional sexual cycle and an active DNA methylation process. The copies of this repeated sequence found today could be relics of RIP consecutive to and fixed at a time where *A. fumigatus* had lost its sexual stage.

Although most researchers have used the PCR- and RFLP-based typing methods separately, a study is under way to compare their discriminatory potential and to evaluate if combination of data obtained by more than one typing method will lead to better strain discrimination (369). To date, strain typing has been most successful by using microsatellite polymorphism or analysis of Southern hybridization patterns obtained with repeated DNA sequences.

CLINICAL SYMPTOMS AND DIAGNOSIS OF RESPIRATORY ASPERGILLOSIS

For most patients, the main portal of entry and site of infection for *A. fumigatus* is the respiratory tract. Although other sites of infections have been described in the normal or immunocompromised host, such as the skin, peritoneum, kidneys, bones, eyes, and gastrointestinal tract, nonrespiratory infections are infrequent and are not discussed here (142, 169, 341, 383, 511). Pulmonary diseases caused by *A. fumigatus* can be classified according to the site of the disease within the respiratory tract and the extent of mycelial colonization or invasion, both of which are influenced by the immunological status of the host (61, 169, 341). Allergic diseases, including asthma, allergic sinusitis, and alveolitis, are not covered in this review. They occur following repeated exposure to conidia or antigens of *Aspergillus* in the absence of mycelial colonization, and in most cases, removal of the patient from the environmental source results in clinical improvement. In contrast, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, and IA, syndromes involving mycelial growth of *A. fumigatus* in the body, usually require therapeutic intervention. The primary symptoms and diagnostic features of these three forms of aspergillosis are described in the following section.

Allergic Bronchopulmonary Aspergillosis

ABPA is currently the most severe allergic pulmonary complication caused by *Aspergillus* species. It occurs in patients suffering from atopic asthma or cystic fibrosis. ABPA occurs in approximately 1 to 2% of asthmatic patients (15% of asthmatic patients sensitized to *A. fumigatus*) and 7 to 35% of cystic fibrosis patients (38, 311, 312, 318, 352, 464). It follows the same course as classic asthma, with a unique cellular immune response and pathophysiologic findings caused by the response of T-cell products (447, 492). Its effects range from asthma to fatal destruction of the lungs with defined clinical, serological, radiological, and pathological features (493, 568).

Clinically, ABPA manifests as a bronchial asthma with transient pulmonary infiltrates that may proceed to proximal bronchiectasis and lung fibrosis (228, 708). It is a very difficult syndrome to diagnose. The criteria classically listed for a definitive diagnosis are the following: asthma, peripheral blood eosinophilia ($>$1,000 mm^{-3}), immediate skin reactivity to *A. fumigatus* antigenic extracts within 15 $\pm$ 5 min, precipitating (immunoglobulin G [IgG] and IgM) and IgE antibodies against *A. fumigatus*, elevated levels of total IgE in serum ($>$1 μg/ml), a history of pulmonary infiltrates, and central bronchiectasis. Less importantly, isolation of *A. fumigatus* from sputum, expectoration of brown plugs containing eosinophils and Charcot-Leyden crystals, and a skin reaction occurring 6 $\pm$ 2h after the application of antigen are also used diagnostically (40, 130, 227, 229, 311, 352, 568, 608, 609, 704).

All the above criteria are rarely fulfilled for each patient with ABPA (226, 227, 601, 746). Moreover, most diagnostic features are not specific and, as a consequence of the intermittent course of the disease, not all criteria are fulfilled at the same time (226, 229, 360). Central bronchiectasis is, for example, detected only in the late stages of the disease (492, 493), and the predictive value of several of these criteria, such as radiographic findings, eosinophilia, or observation of precipitins, may depend on the group (cystic fibrosis patients or asthmatic patients without cystic fibrosis) and age of patients studied (275, 276, 285, 438, 569). The ability to diagnose ABPA would be greatly improved by the use of standardized antigens. Efforts in this direction are being pursued (123, 256). The limitations of the diagnosis of ABPA have led to the concept of "silent" ABPA (598). In some cystic fibrosis patients, for example, damage to the respiratory mucosa in response to exposure to *Aspergillus* conidia occurs even though all of the diagnostic criteria are not met. In untreated patients, ABPA eventually progresses to pulmonary fibrosis and respiratory failure, although some patients have remissions. Obviously, there is a need for improved diagnosis of ABPA and ABPA-related syndromes.

Aspergilloma

Aspergilloma, commonly referred to as "fungus ball," occurs in preexisting pulmonary cavities that were caused by tuberculosis, sarcoidosis, or other bullous lung disorders and in chronically obstructed paranasal sinuses (280, 307, 341, 731). Historically, in the early 1950s, this syndrome was the classical form of aspergillosis. It still occurs today in 10 to 15% of patients with cavitating lung diseases (3). Aspergilloma consists of a spheroid mass of hyphae embedded in a proteinaceous matrix with sporulating structures at the periphery, all of which are found external to the lining of the cavity, i.e., in the airway. A common symptom of aspergilloma is hemoptysis. Hemoptysis results from the disruption of blood vessels in the wall of the cavity occupied by the fungus or in the bronchial artery supply, centimeters away from the aspergilloma (169). Most frequently, internal bleeding occurs, but hemoptysis may be massive and even fatal (3, 101, 128, 190). Aspergillomas appear on chest radiographs as spherical masses usually surrounded by a radiolucent crescent (40, 75). Marked pleural thickening characteristically occurs. High antibody titers (precipitins) are detected in patients with aspergillomas (137, 158, 245, 334, 653). Patients are usually asymptomatic, and aspergillomas are most often detected on chest radiographs obtained for the evaluation of another pulmonary or allergic disease. Today, an increasing number of aspergillomas occur when a solid lesion of IA erodes to the surface of the lung in an immunocompromised host (169). As patients recover from granulocytopenia, cavitation ensues without pleural thickening, and a concomitant increase in anti-*A. fumigatus* antibody occurs. Lesions of this type are best demonstrated by computed tomography (CT) scans of the chest. Their existence should be taken into consideration when the underlying disease relapses or worsens, thereby requiring renewed immunosuppressive therapy (474, 535, 551, 558).

Invasive Aspergillosis

IA has become a leading cause of death, mainly among hematology patients. The average incidence of IA is estimated to be 5 to 25% in patients with acute leukemia, 5 to 10% after allogeneic BMT, and 0.5 to 5% after cytotoxic treatment of blood diseases or autologous BMT and solid-organ transplantation. IA which follows solid-organ transplantation is most common in heart-lung transplant patients (19 to 26%) and is found, in decreasing order, in liver, heart, lung, and kidney recipients (1 to 10%) (119, 221, 489, 533, 682, 718). Although IA is recognized today as the main fungal infection in cancer patients, its true incidence is probably underestimated because of the low sensitivity of diagnostic tests (59, 231, 296, 693). IA also occurs in patients with nonhematogenous underlying conditions; it is increasingly reported in AIDS patients (1 to 12%) (80, 145, 305, 384, 451, 454, 605, 700) and is also a common infectious complication of chronic granulomatous disease (CGD) (25 to 40%) (142, 208). In contrast, it is rarely found in immunocompetent hosts (300).

Four types of IA have been described (142, 682): (i) acute or chronic pulmonary aspergillosis, the most common form of IA;

(ii) tracheobronchitis and obstructive bronchial disease with various degrees of invasion of the mucosa and cartilage as well as pseudomembrane formation, seen predominantly in AIDS patients (145, 305, 454); (iii) acute invasive rhinosinusitis (173, 431, 584, 691, 714); and (iv) disseminated disease commonly involving the brain (10 to 40% in BMT patients) and other organs (for example, the skin, kidneys, heart, and eyes) (59, 481, 532, 729). Clinical features of the different types of IA depend on the organ localization listed above and the underlying disease. These features have been reviewed recently (142, 682) and are not detailed here. However, the diagnostic procedures currently available for IA and their associated problems are discussed. IA remains difficult to diagnose even today, particularly when it is in the early stages. Consensus has not been reached regarding the most appropriate diagnostic criteria for IA. In fact, to prove IA, one must provide histopathological evidence of mycelial growth in tissue. Unfortunately, this is most often demonstrated only at autopsy (64, 231, 682). Moreover, since the hyphae of other filamentous fungi such as *Fusarium* or *Pseudallescheria* spp. may resemble *Aspergillus* spp., definitive identification may require immunohistochemical staining or in situ hybridization techniques (292, 303, 429, 487). Since there is no consensus regarding the criteria used to establish a diagnosis of IA, the terms "highly probable," "probable," "possible," or "suspected" are often used to define IA cases, and definitions vary from study to study.

Features currently considered in the diagnosis of IA include (i) a positive CT scan, (ii) culture and/or microscopic evidence of disease, and (iii) detection of *Aspergillus* antigen(s) in serum. Clinical symptoms are usually too nonspecific to be helpful in narrowing the focus to IA.

As with other forms of aspergillosis, the general symptoms of IA, primarily fever, chest pain, cough, malaise, weight loss, and dyspnea, are variable and nonspecific. The presence of a fever of >38.5°C that is unresponsive to antibacterial therapy, previously recognized as the hallmark for initiating antifungal treatment, is no longer applicable, since corticosteroid-treated patients with IA frequently do not have elevated temperatures (532, 583).

A positive CT scan may be the first definitive suggestion of IA. CT scanning is more sensitive than radiography and shows the extent and number of lesions (75). In the early stages of the infection, CT scans may reveal specific signs of an infection, such as the typical "halo" resulting from hemorrhagic necrosis surrounding the fungal lesion or pleura-based lesions (86, 142, 214, 273, 319, 373–375, 682). Radiographic appearances of pulmonary IA are very heterogenous and can vary from single or multifocal nodules, with and without cavitation, to widespread and large infiltrates which are often bilateral. In nonpulmonary forms of the disease, e.g., rhinosinusitis or cerebral aspergillosis, a CT scan can indicate the extent of the disease and whether bone invasion has occurred. CT scanning can be used in conjunction with brain magnetic resonance imaging in patients with cerebral aspergillosis (532).

The use of culture or microscopic examination of respiratory tract specimens has been criticized because of the presence of airborne conidia of *Aspergillus* and the possibility that a positive culture from such specimens results from accidental contamination (80, 166, 192, 295, 480). The presence of *A. fumigatus* in clinical samples from patients at risk for IA is, however, highly suggestive of an infection, a conclusion which is supported by a careful statistical reassessment of published data (99, 230, 453, 657, 743, 745). In patients with leukemia and BMT for example, microscopic examinations and/or cultures are positive in 50 to 100% of bronchoalveolar lavage fluid (BAL) samples from patients who have definitive or probable aspergillosis (4) and the positive predictive value of a sputum culture in neutropenic or BMT patients has been reported to be >70% (270). The results obtained with BAL samples and sputum samples vary from study to study, however, and some patients have a positive sputum culture and a negative BAL culture or vice versa (270, 453, 743). In some, but not all studies, cultures from nasal swabs of patients were positive repeatedly for *Aspergillus* spp. (4, 6, 399). Bronchoscopy may also provide a suitable specimen for culture, since there is a trend to accept a positive culture from normally sterile sites as a definitive diagnosis for IA (142, 367). Percutaneous lung biopsy specimens or aspirated specimens obtained with radiological or ultrasound guidance, as well as BAL samples, are the specimens of choice. However, since the patient is often quite debilitated, invasive procedures in neutropenic patients demand careful consideration and cannot be repeated.

The recent development of a capture enzyme-linked immunosorbent assay (ELISA) which measures the presence of serum antigens is both sensitive and specific for the diagnosis of IA (345). More information on this topic is given in the next section.

Predisposing factors must be taken into account when assessing the risk of acquiring IA. Because of the difficulty in diagnosing IA and because of its rapid progression (1 to 2 weeks from onset to death) and severity, clinicians often treat the patient empirically rather than waiting for the diagnosis to be established. Moreover, waiting until the diagnosis is confirmed subjects the patient to a greater risk of untreatable IA, since the fungal burden might reach a level too high for antifungal therapy. The extent and duration of neutropenia correlate well with the risk of developing IA. Thus, profound (polymorphonuclear leukocytes [PMN] = 500 mm^{-3} and especially 100 mm^{-3}) and prolonged (>12 to 15 days) neutropenia are associated with the greatest increased risk for pulmonary IA (140–142, 215). Cytomegalovirus infection is also a risk factor for IA in lung transplant recipients but not in BMT patients (85, 274, 532, 696). A major risk factor for all transplant patients is corticosteroid therapy, usually linked to graft-versus-host (GVH) disease and/or rejection in transplantation (696). However, the precise concentration of steroids, as either daily or cumulative doses, associated with the risk of acquisition of IA has not been identified (472, 483). Recently, Ribaud et al. (532) showed that GVH disease and a dose of prednisolone of >1 mg/kg/day for 4 weeks preceding a diagnosis of IA were poor prognostic indicators. A prednisolone dose of 1 mg/kg/day was also noted to be critical for kidney transplant patients to acquire IA (234). In summary, patients at greatest risk for developing IA include (i) allogeneic BMT recipients with prolonged neutropenia or under corticosteroid treatment for GVH disease, (ii) autologous BMT or solid-organ transplant recipients who have been neutropenic for >2 weeks, (iii) patients with acute leukemia and lymphomas undergoing intense chemotherapy, (iv) patients with aplastic anemias and prolonged neutropenia that is nonchemically induced, (v) patients with previously documented aspergillosis subjected to a new chemotherapy regimen or a BMT, (vi) patients with functional neutrophil deficits such as those seen in chronic granulomatous disease (CGD), and (vii) patients with advanced human immunodeficiency virus disease (119, 384, 415, 416, 532, 534, 572, 585, 694, 716, 723, 729).

The risk factors listed above and the severity of the infection illustrate the need for new and prospective methods to diagnose IA. The establishment of such a definition is made difficult by the limited knowledge of the natural history of the disease. For example, the median time to the development of IA is shorter in acute leukemia patients undergoing chemo-

therapy than in BMT patients, in whom IA occurs in 2 to 3 months after transplantation but often with a bimodal symptomatic distribution at 2 to 3 weeks and again 2 to 3 months after transplantation (403, 472, 532, 696). The occurrence of the disease at intervals between 1 week and 2 years after the start of immunosuppression suggests a different pathogenesis, which, in turn, may require the use of different diagnostic strategies. Comprehensive studies which show the relationships among the four criteria mentioned above (general symptoms, CT scanning, culture, and antigenemia) and the underlying disease and immunosuppressive treatment are urgently needed. Improvement in diagnosis should also lead to better management of IA. Of patients at risk for IA, 80% have fever, 40% have fever with pulmonary infiltrates, 25% are treated empirically, and only 6% are definitively diagnosed as having IA (602). A combination of diagnostic strategies is currently being evaluated. For example, when antigen is detected, the disease can be confirmed by performing a CT scan of the lungs and sinuses and radionuclide imaging with ^{111}In-labeled human IgG (602). Compared to the classical strategy for diagnosis (fever refractive to antibacterial agents and the presence of pulmonary infiltrates on chest radiograph), the alternative strategy mentioned above would significantly reduce the number of patients receiving empirical therapy. Finally, the establishment of accurate diagnostic criteria for early symptoms of IA would also lead to a better outcome, since several studies in the last 10 years have shown that reducing the time to obtain a definitive diagnosis was associated with a better prognosis (86, 684, 695).

ANTIGENS AND LABORATORY DIAGNOSIS

Antigens

The antigenic properties of *A. fumigatus* extracts have been recognized for a long time and served as the basis for the early development of immunological assays used in the serological diagnosis of aspergillosis in the immunocompetent host (54, 656). Unfortunately, there are qualitative and quantitative differences in the composition of antigenic extracts prepared in various laboratories and even between batches in the same laboratory (244). In this section, the reasons for the antigenic variability and the most recent approaches to the production of pure antigens of *A. fumigatus* are discussed.

Variability in extracts of *A. fumigatus* does not appear to be related to strain or growth temperature, since the same antigenic pattern has been observed with multiple strains and at both 25 and 37°C (345), but a major source of variability clearly involves other conditions of culture. In particular, the incubation period, the conditions under which the cultures are held, and the composition of the culture medium are critical. There is no standard period of incubation; published periods of incubation have ranged from 1 or 2 days at 25°C with agitation to 5 weeks at 37°C under stationary conditions (244, 334, 376). Different antigenic patterns are produced when the organism is cultured in a defined medium such as Czapek-Dox medium and when it is cultured in a protein hydrolysate medium such as Sabouraud medium. Moreover, the presence of high concentrations of hexose in both media induces an acidic pH during growth and greatly influences the pattern of antigens produced (345, 351, 371, 436). The best complex antigenic preparations are obtained during active fungal growth (1 day at 37°C) in media without sugar but with a single protein substrate or a protein hydrolysate (345, 351). The composition of such a medium appears to be closer to the nutritional environment encountered by the fungus in the lungs, i.e., a protein-rich environment composed primarily of collagen and elastin with a pH close to 7.4.

Other factors that affect antigenic composition include the form of the fungus from which the antigenic mixture is extracted; the method of extraction, including the choice of reagents; and the subcellular source of the antigens (345, 351). With respect to fungal form, although conidial and mycelial (intracellular and extracellular) extracts contain a large number of identical immunologically reactive molecules, multiple qualitative and quantitative differences in their composition can be demonstrated (302, 512, 647). Procedurally, mild extractions, such as a short incubation of intact mycelium in a saline buffer in the presence or absence of a detergent, results in the extraction of loosely associated cell wall components (253, 727). In contrast, cell disruption techniques allow the recovery of all water-soluble mycelial glycoproteins, proteins, and polysaccharides (249, 351). Under these conditions, the choice of disruption buffer is critical as well, in that, e.g., a citrate buffer at pH 4 will solublize different antigens from an ammonium bicarbonate buffer at pH 8. Further, different antigen patterns appear from culture filtrates depending on how the antigenic components are concentrated (351).

In addition to the complications surrounding the extraction of antigens when aspergilli are cultured in vitro under different conditions, there is some evidence that the antigens expressed in vivo during colonization of host tissues are different from those expressed in vitro (83, 156, 581). Since the quantification of antibodies directed specifically against antigens produced in the lung matrix would increase the predictive values of immunological tests, more work must be done in this area.

About 100 proteins or glycoproteins from *A. fumigatus* can bind human Ig, as determined by Western blotting techniques performed after one- or two-dimensional electrophoresis (19, 22, 39, 45, 50, 77, 84, 250, 251, 254, 349, 380, 381, 505, 578, 702). Initially, Western blotting was thought to be the answer to serodiagnostic problems in aspergillosis. Unfortunately, most if not all of these studies have added to the confusion surrounding the antigenic makeup of the fungus for the following reason. The antigenic molecules noted in most of these studies were characterized solely on the basis of molecular mass, which is insufficient to identify an antigen. Consideration must be given to the function of the protein and identification of the encoding gene.

Two examples are illustrative of the problems encountered when studying antigens based only on their molecular mass as identified in Western blots. Three antigens which cannot be separated easily by one-dimensional electrophoresis has been identified in the 90-kDa region, i.e., a catalase (90 kDa), a dipeptidyl peptidase (always present in vitro as a protein doublet of 87 and 88 kDa, each with different levels of glycosylation), and an 88-kDa heat shock protein (41, 83, 89). Biochemical and molecular characterization has been the only way to differentiate these proteins. The second example concerns antigen 7 of Harvey and Longbottom (239). A recent molecular characterization of a homologue of this protein in *A. nidulans* (87) and the use of monospecific antisera (346), as well as careful analysis of previous publications, suggest that the antigens identified as p60, p40, and p37 (380, 381), AspfII (26, 27), gp55 (648), 41 and 53 kDa of CS2 (90, 504), 58 kDa (197), 35 to 65 kDa (333, 338), and GP66 (372) are probably the same protein. Differences in molecular mass can be attributed to the extent of phosphorylation and glycosylation, both of which can alter the size as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and behavior during chromatographic purification. Unfortunately, the isolation of these proteins from different laboratories, using antigenic preparations

TABLE 2. Purified antigenic proteins[a] of *A. fumigatus* reported in the literature

Mol mass (kDa) from SDS-PAGE[f]	Localization[b]	Biochemical function	Gene cloned	Recombinant protein	Reference(s)
12	?	?	+	+	123
18[c]	S	RNase	+	+	21, 126, 168, 343, 344, 349, 377, 424, 433, 434
19	IC	Peroxisomal protein	+	+	123
19[c] (67)[d]	S	Superoxide dismutase	−	NA[e]	236, 267, 268
20	S	?	−	NA	578
27	IC	Superoxide dismutase	+	+	123, 125, 256
28	S	?	−	NA	132
30	?	?	+	+	123
33	S	Serine protease	+	+	287, 424, 428, 435, 436, 520
34	?	?	+	+	123
36 (70?)[d]	S	?	+	+	26, 27, 87, 88, 90, 197, 239, 333, 338, 380, 504, 648
38	S	Aspartic protease	+	+	424, 521–523
40	S	Metalloprotease	+	+	286, 424, 426, 607
82	IC	Metalloprotease	+	−	277
88[c]	S	Dipeptidyl peptidase	+	+	41, 240, 313
90[c] (350)[d]	S	Catalase	+	+	89, 252, 382
93	IC	?	−	NA	243
94	S	Dipeptidyl peptidase	+	+	42

[a] Antigens reacting with antibodies from immunocompetent patients with aspergillosis have been purified by biochemical or molecular biology strategies.
[b] S, secreted (including possible cell wall localization); IC, intracellular.
[c] Most discriminant antigens.
[d] The molecular mass of the native protein is indicated in parentheses.
[e] NA, not applicable.
[f] SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

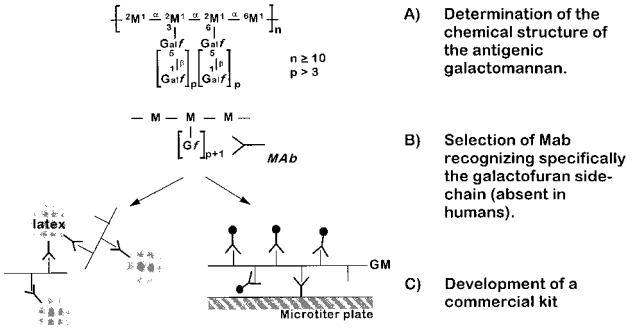
from *A. fumigatus* cultured and purified under different conditions, has confounded the identification of these proteins. Confirmation of identity of antigens with different molecular masses is possible only from protein sequence analysis. Because of this, considerable effort has been expended in the last few years to isolate and characterize to the molecular level polypeptide antigens responsible for specific antibody responses.

Two strategies have been used for molecular characterizations of antigens from *A. fumigatus*. The predominant strategy for antigens shown to be reactive by immunoblotting has been biochemical purification followed by cloning of the structural gene and sequencing (41, 89). A second strategy, however, involves the use of expression libraries to isolate clones which express antigens recognized by patient sera (20, 27, 123, 124, 324). A problem with the latter strategy, however, is that the antigen(s) identified will be highly dependent upon the culture conditions used, since the cDNA library constructed will reflect high-copy-numbers mRNAs expressed by the fungus under those particular culture conditions. By using this strategy, unexpected antigens have been identified, most of them with molecular masses below 40 kDa (123). Only about a dozen of the hundreds of *A. fumigatus* antigenic (glyco)proteins reported in the literature have been characterized at a molecular and biochemical level. They are summarized in Table 2. The most comprehensively characterized antigens, which include an RNase, a catalase, and a dipeptidylpeptidase and the galactomannan, are described in the following section.

The catalase of *A. fumigatus* that has been characterized extensively is a tetrameric protein with a monomeric subunit of 90 kDa (89, 252, 381, 382, 599). The oligomeric subunit contains an N-linked sugar moiety of 7 kDa which bears no antigen epitopes. The protein is remarkably stable, being relatively insensitive to high temperatures, as well as to reducing and denaturing agents. The structural gene for the protein, *CAT1*, has been cloned and sequenced (89). Analysis of the deduced amino acid sequence shows that *CAT1* has both a signal peptide of 15 amino acids and a propeptide of 12 amino acids, with a pair of basic amino acids Arg26-Arg27 acting as a cleavage signal for a KEX2-like endopeptidase. Comparison of the *CAT1* sequence with other catalase genes suggests conservation of the tripeptide His102, Ser141, and Asn175, which is involved in the binding of proteins to its heme prosthetic group.

The dipeptidylpeptidase V has also been characterized recently (41, 313). It releases mainly X-Ala dipeptides and also His-Ser and Ser-Tyr from the N terminus of polypeptides (41). It has a molecular mass of 79 kDa and a signal peptide of 18 amino acids. These data are in agreement with the biochemical data showing that the protein migrates as a doublet of 87 and 88 kDa and contains approximately 9 kDa of N-linked carbohydrate. The biochemical properties, as well as its exocellular localization, indicate that it is an enzyme belonging to a new class of dipeptidylpeptidases (DPPV). Comparison of the *A. fumigatus* DPPV sequence with those of other DPPs shows the presence of a Gly558-X-Ser560-X-Gly562 consensus motif of serine hydrolases with a putative catalytic triad of the DPP arranged as Ser560 Asp643 His675. This protein has no chymotrypsin activity, but it has been referred to as a chymotrypsin antigen (also known as Ag13 or AgC) on the basis of its reactivity resulting in the release of naphthol radicals from a precipitin band when placed in the presence of the chromogenic substrate *N*-acetyl-phenylalanine naphthyl ester (55, 240, 655).

The RNase of *A. fumigatus* is composed of 149 amino acids with a 27-amino-acid leader sequence and a putative active site composed of the six amino acids His49, Glu95, Phe96, Pro98, Arg120, and His136. This RNase cleaves a single phosphodiester bond in a highly conserved region and releases a 300- to 400-base fragment from the 3′ end of the large rRNA (736). It is also known as ASPF1, Ag3, or restrictocin (erroneously named from "*A. restrictus*," since a taxonomical reexamination of the strains used in all recent studies has shown that they are indeed true *A. fumigatus* strains) (19, 343, 349, 377, 434, 735).



D) Comparative evaluation of the tests

	Detection threshold (ng/ml)	Sensitivity (%)	Specificity (%)
Latex agglutination (Pastorex®)[1]	15	25 - 70	90 - 100
Sandwich ELISA (Platelia®)	1	80 - 100	80 - 90

[1] Pastorex and Platelia are registered trademarks of Sanofi-Diagnostic-Pasteur

FIG. 3. Schematic representation of steps involved in the development of a diagnostic test for the detection of antigen in the biological fluids of patients with IA, using GM as an example.

Galactomannan (GM) isolated from cell wall or culture filtrates by a variety of different purification methods has been analyzed (25, 28, 32, 33, 46, 248, 348, 418, 525, 671). It is the only polysaccharide antigen characterized from *A. fumigatus*. Although data differ slightly, a consensus structure has been established: the mannan core has a linear configuration containing α(1-2)- and α(1-6)-linked residues in a ratio of 3:1, and the antigenic, acid-labile side chains, branched on two α(1-2)-linked mannose residues, are composed of β(1–5) galactofuranosyl residues with an average degree of polymerization of 4 (Fig. 3). Numerous intra- and exocellular glycoproteins of *A. fumigatus* with molecular masses of >40 kDa have this galactofuran epitope as well (348). The type of glycosylation involved in the linkage of GM to proteins has not been studied.

Serodiagnosis in the Immunocompetent Patient

Serological testing for the detection of antibodies to *Aspergillus* antigens can be very helpful in the diagnosis of aspergilloma or ABPA, the two forms of aspergillosis observed in immunocompetent individuals. Although growth of the fungus in association with tissue is limited in both of these syndromes, a strong humoral response to the organism frequently occurs (158, 334, 345). Of the more than 20 diagnostic procedures that have been developed to detect anti-*Aspergillus* antibodies, double immunodiffusion and counterimmunoelectrophoresis are the most commonly used in the clinical laboratory (245). These two methods are simple, cheap, easy to perform, and sufficiently insensitive to virtually eliminate false-positive results occurring as a result of the low levels of anti-*Aspergillus* antibodies present in most healthy individuals (246, 345). Historically, these procedures resulted in the discovery of the two major precipitins, the catalase and the dipeptidylpeptidase (the chymotrypsin), which are still used in the serodiagnosis of aspergillosis in immunocompetent hosts (54, 55, 655, 656). The primary disadvantages of the methods are an inability to quantitate the immune response, and lack of standardization due to the use of crude *Aspergillus* extracts (244).

Immunoassays with *A. fumigatus* antigens purified by biochemical procedures have only recently been reported (313, 335, 348, 435, 727). In addition to the difficulty in producing large quantities of pure antigens from in vitro cultures, a minor contamination of even <1% of the antigen of interest with another antigen of greater reactivity may lead to erroneous results (434). To avoid such problems, it is now possible to use molecular biological techniques to produce pure recombinant antigens. For example, proteins of *A. fumigatus* have been produced in *Escherichia coli* or *Pichia pastoris* (41, 88, 89, 434, 435, 607). The *P. pastoris* expression system can yield large quantities of secreted, glycosylated *A. fumigatus* proteins (0.1 to 0.2 mg/ml) (41, 89). Recombinant antigens of *A. fumigatus* reported in the literature (Table 2) are comparable in their antigenicity to the native molecules (41, 88, 89, 123, 126, 256, 433, 435). Such antigens serve as the basis for the development of ELISA methods which will allow the quantitation of the antibody response (245, 246, 313, 349, 727). Studies to select a single antigen or a mixture of antigens that will not only identify the type of aspergillosis but will also have prognostic significance are under way (168, 256, 434, 652). Quantitation of Ig isotypes as well as understanding of the kinetics of the antibody response over the course of the disease will be useful in this regard, since most healthy individuals already have anti-*A. fumigatus* antibodies as the direct result of continuous environmental exposure (278, 301, 309, 339, 349, 376, 660, 679). Since titers in healthy individuals are normally low, infection can be correlated with a rise in specific antibodies. However, selected individuals may have quite high titers owing to occupational exposure or to an underlying disease such as cystic fibrosis, making diagnosis of an infection difficult (123, 256).

Serodiagnosis in the Immunocompromised Host

Circulating antigens. In contrast to immunocompetent hosts, growth of *A. fumigatus* in the tissues of an immunosuppressed host is not correlated with an increase in anti-*Aspergillus* antibody titers. In fact, the presence of anti-*Aspergillus* antibody in immunocompromised individuals is more likely to represent antibody formed before the onset of immunosuppressive therapy rather than as a result of invasive infection. Contradictory data in this regard may be linked to the regimen of immunosuppression used in patient populations (30, 86, 155, 197, 250, 299, 402, 491, 652, 728, 741). An increase in antibody titer at the end of immunosuppression is indicative of recovery from IA, whereas absence of an antibody titer or declining antibody levels suggest a poor prognosis. Thus, antibody detection can be used prognostically but not diagnostically for IA. In fact, the serological diagnosis of IA is based on the detection of circulating antigens in biological fluids, e.g., serum, urine, and BAL fluid, obtained from patients (345). Although the presence of antigens in the serum of patients with IA was first reported in 1979, the number of different antigens identified in the serum or urine remains small (Table 3).

GM was the first antigen detected in experimentally infected animals and in patients with IA (12, 177, 356, 525). Although *A. fumigatus* released large quantities of GM into the culture medium, there is no proof that the GM analyzed from in vitro batches (see above) is identical to the GM circulating in body fluids (347). In vivo, the presence of GM has been demonstrated only indirectly though the use of anti-GM specific antibodies, and its chemical analysis has been hampered by the presence of amounts of antigen (nanograms per milliliter of serum) too small to recover for analysis.

β1-3 glucan, which is another component of the *Aspergillus* cell wall (347), can also be used diagnostically, even though it is not an immunogenic molecule. In this case, the detection

TABLE 3. Molecules detected in biological fluids of patients with IA due to *A. fumigatus*

Antigen	Biological fluid	Detection limit (ng/ml)	Reference(s)
Galactofuran-containing antigens[a]	Serum, urine, BAL fluid	0.5–1	16, 74, 557, 558, 628, 630, 638, 683, 684, 690
29, 18, 11 kDa[b]	Urine	?[c]	241
β(1-3)glucan	Serum	10^{-2}	421, 468, 469, 744

[a] Glycoprotein and polysaccharide.
[b] Plus other minor antigens.
[c] Unknown; detection by immunoblotting.

system is based on the activation of a proteolytic coagulation cascade, whose components are purified from the horseshoe crab (469). A colorimetric assay for detection of β1-3 glucan has been established (421). The components of the assay include factor G, which triggers the β1-3 glucan-sensitive hemolymph-clotting pathway specifically, and a chromogenic Leu-Gly-Arg-pNA tripeptide, which is cleaved by the last component of this proteolytic cascade. The assay can measure picogram amounts of β1-3 glucans and has been used to demonstrate the presence of this polysaccharide during systemic fungal infections (420, 421, 468, 744). The small quantities of β1-3 glucan found in serum can be explained by the fact that β1-3 glucan is an integral component of the cell wall skeleton and, in contrast to GM, is not normally released from the fungal cell.

Few proteins from serum and/or urine of humans or animals infected with *A. fumigatus* have been detected by Western blot assays (241, 503, 742). Different molecular masses have been assigned to the circulating antigens, but only one of these proteins (an 18-kDa protein) has been characterized at the molecular level; it was shown to be ASPF1, one of the major antigens of *A. fumigatus* (241, 293, 349).

Since the discovery of antigens in the serum and urine of patients with IA, the search for antigens in the biological fluids of patients has been presented as the method of choice for the serological diagnosis of IA. However, the detection of antigens has been hampered in the past by the use of insensitive methods (345), which results in a smaller number of positive tests and a delayed diagnosis wherein antigenemia may be detected only one to a few days prior to death. The critical steps necessary to establish a sensitive method for the identification of circulating antigens, using GM detection as an example, are summarized below. Various reagents and assays that have been explored in the development of tests for GM are summarized in Table 4.

The first essential step in the detection of antigen in body fluids is the dissociation of immune complexes (294, 557, 573, 603, 628, 721). Immune complexes result from the normal occurrence of anti-*Aspergillus* antibodies due to continuous environmental exposure in most individuals. Methods currently used to dissociate immune complexes have been selected empirically; additional work is needed to optimize this step, since these treatments can affect antigen detection dramatically. The antibody used in detection can be a polyclonal antibody or a monoclonal antibody (MAb), since the lower limits of antigen detection with both are similar (205, 496, 628). However, the development of a commercial kit requires the use of MAbs because of the disadvantages inherent in polyclonal antisera, such as limited quantities of antiserum and variability from batch to batch. MAbs have been produced against a variety of *A. fumigatus* molecules (19, 22, 65, 198, 201, 204, 323, 329, 524, 621, 629), among which only two have been identified as circulating antigens, i.e., GM and ASPFI (19, 621, 629). The choice of the immunological method used to detect the circulating antigens is a critical step in that the lowest threshold of detection possible must be established. The importance of the detection method is especially well illustrated for the detection of GM. When different methods were evaluated with the same detector MAb, latex agglutination, although very attractive in a commercial context (178, 242, 389, 395) was too insensitive (15 ng/ml) to be useful whereas the recently developed sandwich ELISA system, in which 1 ng of GM/ml of serum is detectable, is suitable (345, 690).

The sandwich ELISA described in Fig. 3 for the detection of GM is currently the most sensitive method developed (628). Several studies performed in Europe have shown that the sandwich ELISA contributes to the early diagnosis of IA, and the inter- and intralaboratory reproducibility of the method is reasonably good (74, 395, 558, 628, 630, 638, 683, 685). In contrast to previous reports, GM was detected in all specimens following the first positive specimen during the course of disease in a given patient. Although it is known that the highest concentration of GM is always released in the terminal phases of the disease, the pharmacokinetics of the antigen in infected animals or humans has been insufficiently studied (47). Depending upon the patient, positive antigenemia can last from 1 week to 2 months (74, 558, 630, 638, 683). GM is detected at a lower concentration in urine, (0.5 ng of GM/ml) than in serum (1 ng of GM/ml) (628). In spite of the lower threshold in urine, and in contrast to previously reported studies (16, 557), the presence of antigen in urine has been shown to be inconsistent, and when present, it did not occur before antigen could be detected in serum (345, 628). Therefore, serum appears to be the most appropriate specimen for the detection of GM in IA. Interestingly, GM can also be detected earlier in BAL samples than in serum, but this sampling method is not always possible in IA patients. Thus, urine or BAL fluid should be secondary specimens in that they are helpful only in confirming a positive serum test.

The ELISA for detection of GM becomes positive at an early stage of infection. The sandwich ELISA was able to detect antigens at least 2 to 3 weeks earlier than the latex agglutination test (242, 558, 690). Early detection is probably the most important feature of these assays, because the detection of antigenemia dictates the initiation of therapy. In some patients, GM was detected in serum before signs and symptoms consistent with IA became apparent (74, 638). Recent studies have shown that IA may be treatable with amphotericin B (AmB) if diagnosed at this stage (74, 558, 638). Another advantage of the ELISA is the possibility that antigen titers in serum can be monitored during treatment. A decrease in the concentration of GM in serum is indicative of treatment efficacy (74, 497, 558, 673).

Despite significant progress in the serological diagnosis of IA by antigen detection, the sensitivity of detection must be improved. The development of an immuno-PCR method for GM (580) or the development of sensitive methods for the detection of other antigens, such as polygalactosamine, which

TABLE 4. Reagents and assays used in the detection of galactofuran-containing antigens in serum of patient with IA

Assay	Detection limit (ng/ml)	Reference(s)
Pretreatment of serum[a]		
Perchloric acid, room temperature		603
Citric acid, boiling		12, 294, 639, 719–721
TCA, boiling		496, 573
PBS, boiling		177, 557, 728
EDTA, boiling		178, 205, 389, 395, 628
Type of Detector Antibody		
Polyclonal antibody	2	205
MAb	1	628
Method of assay		
Latex	15	178, 242, 395, 690
Radioimmunoassay	7	639, 721
ELISA inhibition	5	345, 496, 573
ELISA sandwich	1	628
Ultrasound + latex + videomicroscopy	0.1	232

[a] TCA, trichloroacetic acid; PBS, phosphate-buffered saline.

is present at higher concentrations than GM in cell wall extracts (347), or an as yet unidentified molecule(s) secreted specifically during the early stages of IA may be future answers. Moreover, existing methods have not been evaluated sufficiently. The sensitivity of the method for the detection of β1-3 glucan must be determined, and it must be compared to the ELISA for GM for the early detection of IA. A serious drawback to increasing the sensitivity of a given diagnostic method, however, is the possibility that false-positive rates will increase and will consequently decrease the specificity of the test (630, 634). For example, among the control samples tested by ELISA, an average of 8% false-positive results was found whereas no false-positive results were recorded when the less sensitive latex agglutination method was used (628).

Detection of DNA in specimens. In addition to the detection in body fluids of polysaccharide or protein components of the fungus, it might be possible to develop ultrasensitive PCR-based techniques for the detection of *A. fumigatus* DNA. The data presented in Table 5 support this possibility. Initial studies focused on detection of DNA in BAL samples. Confirmed cases of IA were always associated with a positive PCR test (35, 73, 407, 617, 643). When using PCR, however, extreme care must be taken to avoid false-positive or false-negative results. False-negative results can be monitored by the use of competitive PCR. However, false-positive results are more difficult to control. Since conidia are often present in the air, false-positive results can be generated by the transient presence of aspergilli in the respiratory tract. In fact, up to 25% of BAL samples from healthy subjects are positive by PCR tests (35). In addition, PCR results and GM detection from BAL samples are not congruent (686). Moreover, the number of false-positive samples was higher for PCR assays with BAL samples than for ELISA (73, 628, 630, 688). Recently, very promising PCR results were obtained with serum or plasma (72, 184, 672, 733).

The use of PCR technology with serum or plasma should be pursued, since it has several advantages over the use of BAL samples. First, assuming appropriate handling of the specimen, false-positive results do not occur from environmental contamination. Second, obtaining blood is considerably easier than obtaining BAL fluid. Not only are there technical considerations in obtaining BAL fluid, but also there may be ethical considerations for patients at high risk of IA. Third, sampling can be repeated, so that PCR quantification can be done along with ELISAs. Compared to ELISA, however, PCR positivity seems to occur later than GM detection (72). However, the combined use of PCR and ELISA should result in a definitive diagnosis of IA, even in the absence of obvious clinical signs. Fourth, comparative evaluation of PCR and ELISA data should lead to a better understanding of transient aspergillosis, which may occur in neutropenic patients in the absence of clinical symptoms. Finally, PCR data raise an interesting question as to the origin of the *A. fumigatus* DNA, since the organism is not usually cultured from blood, even in the late stages of disease.

ARE THERE VIRULENCE FACTORS IN *A. FUMIGATUS*?

Strategies

The ideal test for identifying a virulence factor is to compare the infectivity of the fungus in the absence or presence of the factor. Such comparisons have been performed in the past by using naturally occurring mutants or those obtained by UV or chemical mutagenesis (314, 315). The major drawback of these approaches in a fungal species without a sexual stage such as *A. fumigatus* is that the mutant strain may be deficient in more than just the factor being studied. The use of such mutants could lead to an erroneous conclusion about the putative role of the factor studied, as, for example, the proteases (see below).

Molecular biological techniques make it possible to avoid such problems by cloning and disrupting the gene encoding for the putative virulence factor studied. Moreover, the expression of the factor in a heterologous host makes it possible to study its effect in the absence of possible contaminants from the fungus itself, which can occur during any biochemical purification.

Several strategies are available to produce single or multiple mutants of *A. fumigatus* and are summarized in Fig. 4. The classic method involves the disruption of the gene of interest by the insertion of an antibiotic resistance gene. To date, only two genes, one conferring resistance to hygromycin and one conferring resistance to phleomycin, have been used (401, 513). They are placed under the control of either the *GPD* promotor or the *TRP C* terminator of *A. nidulans* or the promotor and terminator of the gene subjected to disruption (427, 486). Disruption is usually made in a nitrate reductase-deficient background to take into account the possibility of external contamination. However, these systems can lead to only two successive mutations (286). To compensate for this disadvantage, a *PYRG* blaster has been developed recently in our laboratory (138). This system is very similar to the *URA* blaster previously developed in *Saccharomyces cerevisiae* and *Candida albicans* (194). The system consists of the *A. niger PYRG* gene flanked by a direct repeat that encodes the neomycin phosphotransferase of Tn*5*. The *PYRG* cassette is inserted by gene replacement following transformation of a uridine/uracil-auxotrophic *PYRG* strain. Recombination is selected in the presence of 5-fluoroorotic acid, which results in the excision of the *A. niger PYRG* gene, producing *A. fumigatus* uridine/uracil auxotrophs which have retained their mutant phenotype because of the persistence of one of the two elements of the direct repeat at the site of insertion of the *PYRG* blaster. Selection for uridine/uracil prototrophy can be used again to disrupt another gene.

TABLE 5. Analysis of PCR results in patients with and without IA

Specimen	Probe	Total no. of patients	No. positive		Reference
			PCR^+/IA^{+a}	PCR^+/IA^{-b}	
Urine	18-kDa ribotoxin	13[c]	1/1	1/12	519
BAL fluid	rDNA	10[d]	3/3	2/7	617
BAL fluid	33-kDa alkaline protease	51	5/5	6/46	643
BAL fluid	rRNA	25	6/6	0/19	407
BAL fluid	rRNA	70	NA[h]	11/70	688
BAL fluid	mtDNA	52	3/3	12/49	73
Serum	mtDNA	23[e]	5/5	0/18	72
Serum	rRNA	40[f]	14/20	0/20	733
Plasma	rRNA	77	13/13	0/64	184
Plasma	rRNA	8[g]	3/3	0/5	672

[a] Proven or highly probable disease based on clinical data.
[b] Healthy donor or patient without any clinical signs of IA.
[c] Another urine sample was PCR positive, but no clinical data were given for the patient.
[d] Thirteen other patients were analyzed, but clinical data were insufficient to classify.
[e] Nineteen patients were positive for GM.
[f] Twelve patients were positive for GM by the latex agglutination test.
[g] A total of 3 of 189 blood samples from 103 control patients gave false-positive results.
[h] NA, not applicable.

Transformation can be performed with protoplasts or by electroporation (78, 486, 717).

Another possible approach to understanding virulence in *A. fumigatus* is the construction of libraries of mutants through random insertional mutagenesis (78, 79, 258, 717). Conditions for restriction enzyme-mediated integration (REMI) have been recently published; *Xho*I or *Kpn*I digestion was used to obtain a single-copy integration of transforming DNA with the majority of the transformants (78, 266). The signature-tagged mutagenesis approach developed for bacteria has also been applied recently to *A. fumigatus* (79, 266). Mutations which will render strains avirulent will allow for the cloning of the virulence genes disrupted by the mutagenesis.

Animal Models

The second essential tool, in addition to gene disruption techniques, in the identification of a virulence factor is an appropriate animal model in which to test virulence in vivo. Invasive pulmonary aspergillosis has been established in mice, rabbits, rats, guinea pigs, chickens, cows, turkeys, ducks, and monkeys (96, 171, 289, 291, 293, 392, 507, 536, 674). Originally, the animal models were developed to study the efficacy of antifungal drugs in the treatment of aspergillosis (8, 14, 196, 224, 495, 596, 673) or to evaluate diagnostic methods (177, 178, 497, 540, 720). There is no consensus about the best model to use. Indeed, a survey of the literature reveals that there is variation among researchers not only with respect to the choice of an animal (strain, weight, and sex) and immunosuppressive regimen (dose, products, frequency of the injections) but also with respect to the challenge protocol (concentration of conidia and route of injection). In spite of the heterogeneity in the animal models used, however, several conclusions can be drawn.

(i) As with other fungal pathogens, there is a direct relationship between dosage of conidia and lethality. The weight of the animal is critical as well; for any given species, heavier animals require larger dosages of conidia to establish disease (116, 171).

(ii) Immunosuppressive treatments substantially increase the susceptibility of animals to infection, and striking differences in the patterns of infection and inflammation in IA are related to the type of immunosuppression used (48) (Fig. 5). In rabbits, profound granulocytopenia initiated with cytosine arabinoside resulted in more severe IA than when immunosuppression was induced by cyclosporin A plus methylprednisolone (8, 48, 196). Because they are easier to use, cortisone and cyclophosphamide are preferred for immunosuppression in IA in mice and in rabbits (102, 494, 619). Multiple injections of the drugs should be performed with care since they result in increased mortality in controls unless the animals are housed under sterile conditions and fed sterile food and drinking water supplemented with antibiotics. Pregnant animals develop IA in the absence of immunosuppressive treatment (290, 291). A low-protein diet favored the development of experimental aspergillosis in rodents (463).

(iii) Mice, especially outbred Swiss mice, have been the most common animals used. Most mouse strains, regardless of genetic background, are equally susceptible to *A. fumigatus* (93, 94, 171, 612). Athymic nude mouse (726) are no more susceptible than inbred C57BL/6, BALB/c, or CD2F1 mice. Only C5-deficient mice, such as DBA2, have been reported to be extremely susceptible to *A. fumigatus* infection, regardless of the portal of entry of conidia (93, 94, 255). This particular difference in host sensitivity may be useful in the study of the fungal pathogenesis. In this context, the selection of transgenic mouse strains with different levels of susceptibility to *A. fumigatus* will be essential to our understanding of the infection process (120, 337, 404, 432).

(iv) Intranasal (i.n.) inoculation mimics the natural route of infection and would seem to be a more appropriate route than intravenous (i.v.) inoculation. Following inhalation of conidia and invasion of the lungs, the fungus disseminates to other organs such as the brain, liver, kidneys, and spleen. As in humans, disease develops only if the animal is immunosuppressed, since an immunocompetent mouse is able to clear as many as 10^8 conidia without developing disease (165). The major drawback of the model is the highly variable response of animals to a given inoculum, necessitating the use of large groups within each experiment. In contrast, i.v. challenge incites a more uniform pattern of disease, but the mode of infection and the primary infected organ, i.e., kidneys and brain in nonpregnant animals and placenta in pregnant animals, are quite different from the human infection (289, 292). In addition, IA can be produced as the result of i.v. challenge in the absence of immunosuppressive treatments, a situation

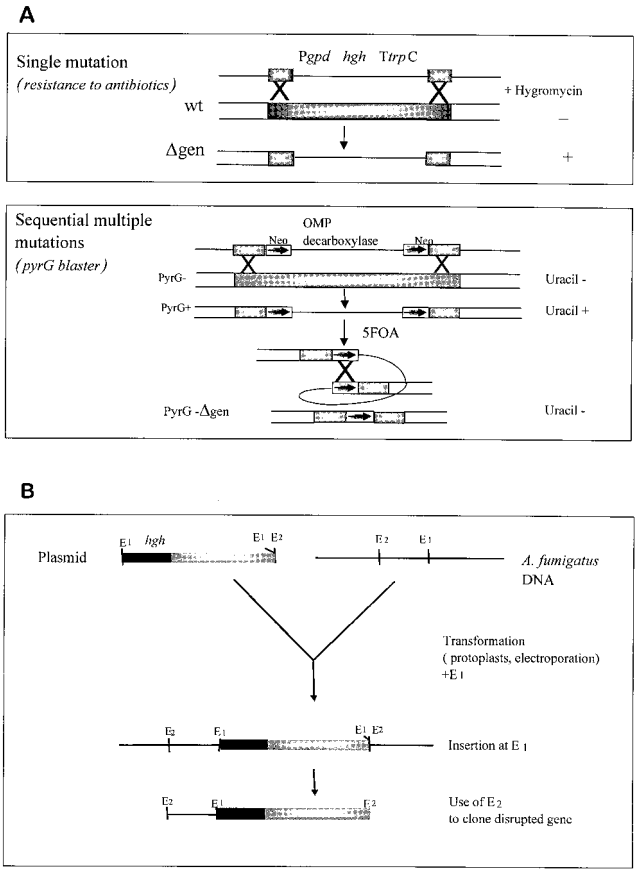


FIG. 4. Strategies currently available to disrupt genes in *A. fumigatus* (A) and to create a bank of *A. fumigatus* mutants by using the restriction enzyme-mediated integration strategy (B). E1 and E2 are endonucleases favoring a single-copy integration of the marker in the *A. fumigatus* genome. wt, wild type; OMP, orotidine-5′-phosphate; 5FOA, 5-fluoroorotic acid.

which is different from the development of IA in humans or in mice challenged i.n. (94, 116, 177, 581, 612, 720).

For these reasons, i.n. challenges are recommended for the study of pathogenesis, and several systems have been used for the experimental inhalation of conidia. In one of the older methods, a chamber in which mice received aerosols of conidia was used (591, 641). This method works, but it is time-intensive and not useful for uniform infection of large numbers of animals. Similarly, intratracheal challenge by direct instillation of the conidial suspension into the respiratory tract is impossible to use with a large number of animals. The easiest method of inoculation is the i.n. instillation of 25 μl of a suspension of conidia to anesthesized mice. The number of conidia required to kill mice, which depends on the immunosuppressive regimen and the route of inoculation, is shown in Fig. 5.

(v) Due to the extensive development of mycelium during disease and the long-term survival of ungerminated conidia in the lung, quantitation of fungus by CFU measurement does not represent a good estimate of the severity of the disease. In contrast, chitin determination, which correlates well with fungal biomass, may more accurately reflect growth in vivo (94, 157, 358).

(vi) In the usual approach to the study of virulence, strains of *A. fumigatus* are inoculated into separate groups of animals and death is monitored over time. It was shown in two studies of this type that the virulence of wild-type, clinical, and environmental strains varied according to the source (315, 423). Increased virulence of clinical strains was associated in one study with the presence of an uncharacterized 0.95-kb DNA fragment (423). Models involving mixtures of strains may be more useful to quantify variations in strain aggressiveness that are not detected when isolates are studied individually. For example, when six wild-type strains were mixed and inoculated in a single challenge, one or two of the strains in the inoculum were not reisolated from the lungs of dead mice, suggesting that these strains were unable to compete in that environment. In contrast, when each of the six strains was inoculated i.n. separately in corticosteroid-treated mice, they were able to kill mice without significant differences in survival data (582). Interestingly, differences in virulence, which do not represent additive effects, can be also shown when a mixture of mutant strains is inoculated into mice at the same time (350, 644). Infection with pools of numerous strains is an approach which makes possible the screening of thousands of tagged randomly generated mutants and the putative identification of avirulent strains (78, 79, 258, 614).

(vii) The major drawback to animal models is the lack of conformity to the human infection. Experimentally induced aspergillosis is a hyperacute infection obtained with a high concentration of conidia given at a single point in time. In humans, IA is often a more indolent disease, caused by continuous or repeated exposure to small numbers of conidia. Infected rabbits die within 5 to 10 days after challenge with a suitable inoculum, whereas mice die 4 to 7 days after infection. Two new approaches have been introduced to improve animal models. First, the inoculum size can be reduced if the severity of the immunosuppression is increased by using cyclophosphamide more frequently (614). The drawback to this approach is the susceptibility of the immunosuppressed mice to other infections and the necessity to keep the mice in a sterile environment. Second, a new model has been developed by Nawada et al. (455), wherein intratracheal administration of agarose beads coated with *Aspergillus* conidia is followed by pulmonary aspergillosis. In this model, the fungus remained alive for at least 6 weeks before the immunosuppressive treatment was introduced to induce IA.

In spite of an urgent need, there is still no animal model for chronic IA. Transgenic mice may be the solution for the establishment of such a model. Mice with X-linked CGD generated by targeted disruption of the $gp91^{phox}$ subunit of the NADPH oxidase complex may be one example (432). Pulmonary disease was observed in the transgenic mice after administration of doses as low as 50 conidia per mouse with the subsequent development of chronic inflammatory lesions.

(viii) A murine model of ABPA with eosinophilia and elevated IgE levels has been developed and has proven useful for the study of that clinical syndrome (107, 326, 336, 707). In contrast to invasive disease, this model relies on the repeated inoculation (twice a week for 4 to 8 weeks or three times a week for 3 weeks) of nonviable antigenic extracts. The mode of inoculation (intraperitoneal or i.n.) and the form of the antigen inoculated (soluble or particulate) determine the immune response and pathological findings: animals exposed by the i.n. route to soluble antigens exhibited significantly higher IgE and IgG levels. Blood and lung eosinophilia was more pronounced in animals exposed i.n. to particulate antigens than soluble antigens and was absent after an intraperitoneal challenge (327, 340).

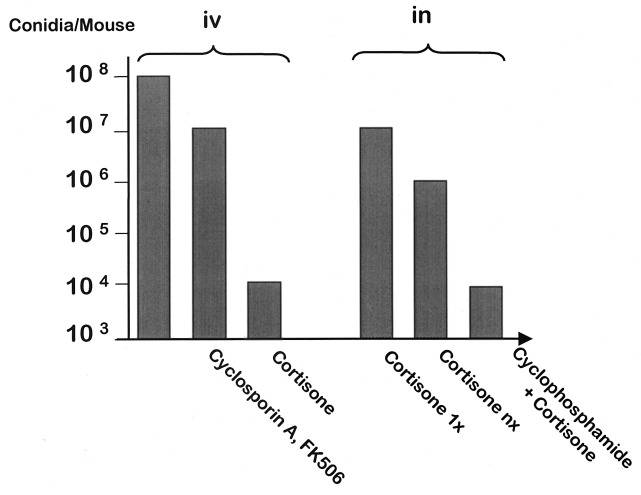


FIG. 5. Effect of different immunosuppressive drugs and routes of infection on the number of conidia required to induce IA in outbred Swiss mice. Data from references 94, 110, 111, 116, 171, 224, 264, 423, 540, 581, 612, 614, 641, and 649.

Putative Virulence Factors

To invade the host, *A. fumigatus* must be able to adhere to and penetrate the human respiratory epithelia and kill the surrounding cells, in particular phagocytic cells that are actively involved in defense against *A. fumigatus* (350, 351). The adhesins, hydrolases, and toxic molecules of *A. fumigatus* which are putatively involved in this invasive process and which have been characterized to date are listed in Table 6.

Adhesins. Conidia bind specifically to various circulating or basement membrane-associated host proteins: fibrinogen, laminin, complement, fibronectin, albumin, Igs, collagen, and surfactant proteins (15, 67, 68, 76, 122, 216, 391, 499, 626, 649, 661–664). Binding occurs through nonspecific physicochemical interactions and/or specific receptor-ligand recognition. Only a few of the existing adhesion systems have been characterized in *A. fumigatus* to a biochemical and/or molecular level.

The outermost cell wall layer of *A. fumigatus* conidia is characterized by the presence of interwoven rodlet fascicles. This layer, which is composed of hydrophobic proteins (hydrophobins), confers hydrophobic properties to *A. fumigatus* conidia (649). The hydrophobins are a family of homologous proteins that are present on the surface of dry conidia of different fungal species (722). All hydrophobins have eight conserved cysteine residues, similar hydropathic patterns, and low molecular mass in the range of 10 to 20 kDa and are extremely resistant to chemical degradation, which allows their extraction by concentrated acids. The gene encoding the rodlet protein RodA of *A. fumigatus* has been cloned, and a rodletless mutant has been generated (488, 649). Conidia from this mutant were hydrophilic and bound less readily to proteins with hydrophobic pockets such as albumin or collagen (649). Binding to other proteins such as laminin and fibrinogen was not altered in the mutant. In animal models of IA, mortality was comparable for the parent and rodlet-less strains but the inflammatory response was lower with the Δ*rod*A mutant (606, 649). Thus, RodAp appears to play an essential role in the hydrophobic interactions between the fungus and the host, but these interactions do not appear to be essential for the virulence of the fungus. Other hydrophobic proteins have been detected in *A. fumigatus*. The nature and role of other hydrophobic proteins produced by germinating conidia and the mycelium of *A. fumigatus* are not known (498), but one such conidial protein of 14 kDa, which coextracts with the 16 kDa RodA protein (484), is under study in our laboratory.

Carbohydrate and protein molecules on the conidial surface are involved in binding to host proteins in a specific and saturable manner. For example, an unknown carbohydrate molecule on conidia bound in a calcium-dependent manner to pulmonary surfactant proteins A and D (391). Fucose- and sialic acid-specific lectins also have been identified on the conidial cell wall (68, 279). Western blot analyses have shown that complement binds to a 54- and 58-kDa doublet protein found on the surface of the conidia (626). However, in contrast to *Candida* spp., complement receptors have not been investigated in *A. fumigatus* (317, 626). The receptor for laminin is a 72-kDa glycoprotein also present on the surface of the conidium (664). Binding of fibrinogen, laminin, fibronectin, and complement is associated with the outer and inner wall layers of the conidia with a different localization for each protein, suggesting that different *Aspergillus* proteins bind to unique host proteins (663). None of the specific *A. fumigatus* adhesins has been purified to date, and their role in the establishment of disease will remain debatable until a mutant devoid of adhesive capacity is obtained.

Pigments. Wild-type strains of *A. fumigatus* lacking pigment are less pathogenic than strains with green conidia (350, 582). Two genes, *ALB1* and *ARP1*, encoding, respectively, a polyketide synthase and a scytalone dehydratase, which are involved in the biosynthesis of the dihydroxynaphtalene melanin, have been recently cloned and disrupted (665, 666). The use of isogenic nonpigmented mutants has confirmed the data obtained with the wild-type nonpigmented strains (283, 665, 666). White *A. fumigatus* conidia bind more avidly to complement (666) and their wall seems to be more permeable, as shown by an increased susceptibility to antifungal drugs (689). These results are reminiscent of previous studies performed with dematiaceous fungi or the melanin-producing yeast *Cryptococcus neoformans*, where melanin has been shown to protect cells from human host defenses and increase the in vivo survival of such strains (265). However, such results have minor implications in the control of the disease since almost all wild-type conidia are green and the invading mycelium hyaline. Besides, nonpigmented conidia inoculated into steroid-treated mice were still able to produce IA.

Toxic Molecules. *A. fumigatus* produces several toxic secondary metabolites (129, 200, 667). The most intensively studied is gliotoxin, a metabolite of the epipolythiodioxopiperazine family (439, 442). This compound, which is acutely toxic (195), also has a broad range of immunosuppressive properties. Because of these properties, gliotoxin was once considered useful as immunosuppressive therapy for BMT patients (440, 441)! Gliotoxin inhibits macrophage phagocytosis and induces apoptosis in macrophages by a mechanism distinct from its antiphagocytic properties. It also blocks T- and B-cell activation and the generation of cytotoxic cells (182, 439, 442, 632, 633, 709). Gliotoxin has been detected in infected animals and humans at concentrations at least as high as that required to see an effect in vitro (183, 536). Other secondary metabolites can impair the mucociliary action and result in prolonged residence of the fungus at the surface of the epithelium (9, 10, 113). The effect of these toxic secondary metabolites could favor invasion by *A. fumigatus*. However, their role remains questionable since (i) species of *Aspergillus* which do not produce them can be pathogenic and (ii) null mutants have not been constructed due to our lack of knowledge of the enzymes of the metabolic pathway responsible for the production of these secondary metabolites in *A. fumigatus*.

TABLE 6. *A. fumigatus* molecules with a putative role in virulence

Category	Role in vivo	Molecule	Reference(s)
Adhesins	Promotion of interactions of host proteins and cells with *A. fumigatus*	Complement receptor (54–58 kDa)	626
		Laminin receptor (72 kDa)	664
		Hydrophobins (14 and 16 kDa)	484, 649
Pigments	Inhibition of phagocytosis of conidia	Dihydroxynaphthalene-melanin	284, 350, 666, 667
Toxic molecules	Host cell death	RNase (18 kDa)	19, 189, 343, 344
	Erythrocyte lysis	Hemolysin (30 kDa)	179–181, 206, 207, 737–740
	Immunosuppression	Secondary metabolites, e.g., gliotoxin	10, 129, 182, 632, 633, 709
Enzymes	Promotion of lung matrix colonization and/or degradation of humoral factors	Serine protease (33 kDa)	427, 428, 641
		Aspartic protease (38 kDa)	521, 522
		Metalloprotease (40 kDa)	286, 426
		Dipeptidylpeptidases (88 and 94 kDa)	41, 42
	Antioxidants during phagocytosis	Catalases (350 kDa and unknown)	89
		Superoxide dismutases (27 and 67 kDa)	125, 267, 268
	Epithelial damage	Phospholipase(s)	57

In addition to toxic secondary metabolites, *A. fumigatus* produces an 18-kDa RNase (19, 21, 335, 343) and a 30-kDa hemolysin (179–181, 206, 207, 737–740), both of which have toxic effects. Of the two proteins, the 18-kDa RNase, known as ASPF1 or restrictocin (see above, antigen section), has been studied more extensively. The RNase cleaves the 28S rRNA of eukaryotic ribosomes at a very specific and universally conserved 14-nucleotide purine-rich sequence (189, 736). Modeling studies of the crystalline structure of restrictocin suggest that the tertiary structure of the substrate RNA is important in protein-RNA recognition. In addition, a large 39-residue loop, which has similarity to loops found in lectin sugar-binding domains, may be responsible for the ability of this protein to cross cell membranes (736). Among its other features, it is present in the conidial cell wall and is secreted in vivo, as shown by its detection in the urine of patients with IA and in the regions of necrosis surrounding the fungus in the tissues. It has extremely powerful toxic effects at nanomolar concentrations in an immunoconjugate form or on cells with altered permeability, whereas killing of unmodified cells occurs at micromolar concentrations (344, 349, 390, 445, 715). However, there are several observations which argue against a major role for this molecule in the pathogenicity of *A. fumigatus* infections: (i) other medically important species of *Aspergillus*, including *A. flavus*, do not synthesize this molecule, whereas some nonpathogenic species do produce it (351); (ii) injection of milligram amounts of this protein into animals did not result in toxic shock (349), and (iii) a mutant constructed by disruption of the *ASPF1* gene was indistinguishable from the parental strain in its growth in lung tissue and mortality of mice (486, 613).

Understanding the exact nature of the effect of the toxic molecules may be more complicated or subtle than was originally thought, however. Preliminary experiments have shown a positive cumulative toxic effect when mixed lymphocyte populations were treated sequentially with subinhibitory doses of gliotoxin and Aspf1. In addition, a mutant Aspf1p (His136→Leu136), which does not display RNase activity but which retains antigenicity, stimulated the immune response to a greater extent than did the native toxic Aspf1p (134, 735). Such experiments illustrate that the effect of a putative virulence factor may be different when it is tested alone from when it is tested in combination with other *Aspergillus* molecules and that two functions displayed by the same molecule can interfere and stimulate different host responses.

The hemolysin of *A. fumigatus* is the only hemolytic compound whose activity is inhibited by apolipoprotein B (207). It contains a set of negatively charged domains similar to portions of the cysteine-rich sequence in the ligand-binding domain of the low-density lipoprotein receptor (180). This hemolysin has been detected in vivo in experimental *Aspergillus* infections. The multiple biological functions attributed to this protein may enhance the development of *A. fumigatus* infections (181, 207, 739), but the definitive experiments to prove it have not been done.

Enzymes. It is logical to assume that selected enzymes are essential for any fungal pathogen to invade host tissue (265, 470). Since the lung matrix is composed primarily of elastin and collagen, it was expected that elastinolytic and collagenolytic activities would play a major role in infection (425). Indeed, several studies have suggested the possible involvement of proteases in the pathogenesis of aspergillosis: (i) wild-type strains exhibiting a high proteolytic activity in vitro were more pathogenic in an animal model than were strains with low proteolytic activity (315); (ii) comparison of clinical and environmental isolates of *A. fumigatus* showed a higher proportion of protease producers among the clinical isolates (531); (iii) a protease-negative strain obtained by chemical mutagenesis was less pathogenic than the parental strain in an animal model of aspergillosis (314); (iv) protease induced the release of proinflammatory cytokines (interleukin-8 [IL-8], IL-6, and monocyte chemotactic protein 1) and caused cell detachment in a human pulmonary cell line (654); and (v) protease secretion by *A. fumigatus* can be demonstrated in the infected lung by com-

plementary observations, as follows. First, several authors have used anti-protease-specific antibodies to label thin sections or perform Western blotting from infected tissues (314, 397, 436, 520). Second, a fusion gene consisting of the promoter of the alkaline protease *ALP* and the *Escherichia coli lacZ* gene was used to demonstrate histochemically that the *ALP* promoter was activated during growth in the lungs (504). Finally, antibodies against the three major proteases identified to date, are present in sera of patients suffering from aspergilloma (426, 428, 521).

The major protease secreted by *A. fumigatus*, when cells are incubated with a protein or protein hydrolysate at neutral pH, is a serine alkaline protease (Alpp) of 33 kDa. Alpp is a member of the subtilisin family and has been extensively characterized both biochemically and genetically (202, 287, 314, 428, 520). However, gene disruption experiments have shown that both isogenic Alpp-producing wild-type and Alpp-nonproducing strains cause the same mortality in a murine model of IA (427, 614, 640, 641). The Δ*alp* mutant was not able to degrade elastin but retained the ability to lyse collagen (426). Of the total collagenolytic activity, 30% was due to a 40-kDa metalloprotease (Mepp) of the neutral protease I family (397, 425, 426, 607).

The *MEP* gene has been cloned and disrupted in both wild-type and Δ*alp* strains (286, 607). The double mutant Δ*alp* Δ*mep*, which does not express neutral proteolytic activity extracellularly in vitro, is still able to infect and kill immunosuppressed mice in a manner similar to the wild-type cells (286). Although a total lack of extracellular proteolytic activity was demonstrated for the Δ*alp* Δ*mep* strain at neutral pH in vitro, one can still hypothesize that additional proteases were produced in vivo by induction with specific host factors. Other proteases, although not expressed or expressed at low levels in vitro, might compensate in vivo for reduced proteolytic activity due to the disruption of the genes encoding the major proteases (396, 515). Putative candidates for such a protease(s) are those of the neural protease II class (516) or homologs of the *ALP* product which have been identified by low-stringency Southern hybridization (259, 614).

Recently, a 38-kDa protease (Pepp) of *A. fumigatus*, belonging to the pepsin family of aspartic proteases, has been characterized (353, 521–523). Although Pepp has been demonstrated in human lung tissue infected with *A. fumigatus*, in contrast to Alpp and Mepp, its expression does not seem to be induced by collagen or elastin. Pepp-deficient mutants invaded tissues to a similar extent to the parental strain and produced comparable mortality in guinea pigs (522), suggesting that Pepp, like Mepp and Alpp, does not contribute to tissue invasion in systemic aspergillosis. In addition, the occurrence of Pepp in the sporulating structure of *A. fumigatus* and the discovery of a protein tightly bound to the cell wall which cross-reacts with an anti-Pepp antiserum suggest that this family of aspartic proteinases of *A. fumigatus* may play a role only in fungal morphogenesis.

Arguments against a role for proteases in host invasion have been proposed as well: (i) a Δ*are*A mutant with significant proteolytic activity in vitro when incubated in a medium containing collagen as the sole carbon and nitrogen source caused less mortality than the parental strain did (259); (ii) histopathological studies have shown that in the lung tissue of humans or experimental animals, the collagen and elastin matrix is traversed without any apparent proteolytic degradation associated with the invading fungus (153, 286); (iii) a MAb which inhibited the elastase of *A. fumigatus* did not reduce the virulence of the fungus in immunocompromised mice (201, 203); and (iv) proteases, highly homologous to the enzymes found in *A. fumigatus* are produced by species which are rarely the causative agents of IA, such as *A. oryzae*, *A. sojae*, or *A. niger* (425).

The analyses of *Aspergillus* proteases will probably continue, but at present there is no proof that any protease of *A. fumigatus* plays an essential or a specific role in the pathogenesis of IA. The function of these enzymes may be to allow the fungus to degrade dead animal tissue only for use of that tissue as a nutritional substrate and therefore to compete successfully with other saprophytic microflora.

Other enzymes listed in Table 6 have been isolated, and some are described in the section on antigens (see above). While their role in pathogenicity has just begun to be tested, the oxidative enzymes may play an essential role during the infectious process. As shown with other pathogenic microorganisms, such enzymes can counteract the killing effect of reactive oxygen species produced by the phagocytic cells. Currently, three catalases and two superoxide dismutases have been found in *A. fumigatus* (89, 125, 252, 267, 268). Demonstration of oxidases as putative virulence factors, however, is complicated by the fact that there are multiple oxidases with redundant function. Indeed, the gene encoding the antigenic exocellular catalase *CAT1* has been disrupted recently without any sign of increased susceptibility of the Δ*cat*1 mutant to oxidative stress in vitro and in vivo (89).

The assessment of the role of an enzymatic activity such as catalase will require multiple disruption events. However, as with the proteases described above, strains lacking oxidase activity in vitro may retain virulence, since the secretion of other proteins with similar activity but expressed specifically during colonization of lung tissue may occur. For example, *A. niger* has four catalases, two of which are produced only under conditions of stress (730). Another complicating factor is the absence of additivity of gene expression in assays for pathogenicity. For example, although the in vitro catalase activity is quite reduced in Δ*cat*1 single catalase disruptants and diminished even further in Δ*cat*1 Δ*cat*2 double catalase disruptants, no difference in virulence was seen between the single and double mutants and the parental strains in an experimental model of IA (484). Similar results have recently been obtained with single and double catalase disruptants of *A. nidulans*, which are as pathogenic as the parental strains in both CGD transgenic mice and cortisone-treated BALB/c mice (95).

In recent years, molecular biologists have postulated that enzymes which play a key biosynthetic role for *A. fumigatus* should be considered as possible virulence factors. In fact, mutations in genes encoding enzymes regulating the metabolism of *p*-aminobenzoic acid and pyrimidine result in auxotrophy, and such mutants were unable to germinate in vivo where these metabolites are not freely available (139, 644). Consequently, they are avirulent. The use of the term "virulence factor," however, should not be applied to such enzymes but only to factors which do not block morphogenesis in vitro. The use of mutants of this type can be useful in monitoring the survival of the fungus as resting conidia in immunocompromised hosts, since these conidia will germinate as soon as the metabolite is added to the drinking water of mice infected with the auxotroph. Growth rate may indeed be a key determinant in the progression of invasive aspergillosis and therefore of *A. fumigatus* pathogenicity (460). For example, a mutant deficient in *CHSG*, a gene of the chitin synthase family of *A. fumigatus*, has a reduced radial growth rate and also has reduced virulence (408). However, other cell wall mutants with altered mycelial growth caused comparable mortality to the parental strain, suggesting that the role of hyphal branching and growth rate in the establishment of the disease remains debatable (24,

437). The small size of the conidia has been suggested to be a key factor in the pathogenicity of *A. fumigatus* and has been claimed to be responsible for the lower virulence of *A. niger*, which has conidia with diameters of 5 μm (165, 611). However, the data are too limited at present to support this conclusion.

All of the data accumulated to date suggest that the virulence of this fungus is multifactorial. If such a hypothesis is valid, a strategy based on single-gene deletion experiments is not appropriate for the study of the virulence of this fungus, unless a mutagenized regulatory gene controlling a cluster of genes involved in pathogenesis is identified. For example, revertants suppressing the Δ*areA* low-virulence phenotype, which is associated with a delayed onset of IA symptoms in mice, have been obtained (259). Selection of these revertants should allow the identification of components or metabolic cascades controlled by *AREA*, a transcription factor regulating nitrogen metabolism, which are essential to the pathogenicity of the fungus in vivo (257, 259).

With the exception of melanin, no other virulence factors have been identified in *A. fumigatus* by the single-gene strategy mentioned above, and new approaches must be designed. For example, differential expression of genes in vivo has not been studied in *A. fumigatus*, although several methods developed for the study of bacterial pathogenesis such as in vivo expression technology (393) could be applied directly to the study of *A. fumigatus* infection. In addition, the lack of identification of fungal factors specifically expressed in vivo and the absence of truly avirulent strains, as opposed to strains with reduced virulence, make comparative or complementation studies difficult. However, the difference in the aggressiveness of wild-type strains observed when several strains were inoculated together into immunocompromised mice (350) suggests that a collection of wild-type strains with attenuated virulence could be found or that mutants could be constructed genetically by random mutation. The virulence of this fungal species may involve resistance to the antifungal mechanisms of the human host rather than to the expression of specific disease-related proteins. The melanin data, in fact, suggest that *A. fumigatus* becomes a pathogen because it resists host defenses and survives in vivo longer than other airborne saprophytes (443). Identifying the factors responsible for the resistance of *A. fumigatus* in vivo requires, as a prerequisite, a thorough understanding of the defense mechanisms displayed by the human host against this fungal species.

HOST DEFENSE MECHANISMS AGAINST *A. FUMIGATUS*

Innate Immunity

Nonspecific or natural immunity plays a major role in the defense against *A. fumigatus* by recognition and clearance of the organism in immunocompetent hosts (586–588). Immunocompetent laboratory animals challenged with high doses of conidia eliminate the majority of the inoculum in a few hours. Elimination curves follow first-order kinetics, suggesting that innate defense mechanisms play a major role in the clearance of conidia. Nonspecific immunity includes three major lines of defense: (i) anatomical barriers, (ii) humoral factors such as complement, and (iii) phagocytic cells and their related antimicrobial products. Each is discussed below with respect to the development of aspergillosis.

Anatomical barriers. The majority of the conidia of *A. fumigatus*, like most airborne particles, are probably excluded from the lungs through the ciliary action of the mucous epithelium. However, the clearance of *A. fumigatus* at this level may be less efficient than with other airborne saprophytic microorganisms, since toxic molecules produced by *A. fumigatus* inhibit ciliary activity and since proteases can damage the epithelial tissue (10, 552). In addition, epithelial and endothelial cells have recently been shown to internalize conidia, serving as putative foci of infection (136, 485, 646, 650). In animal models, penetration at the epithelial level is common (139, 185). In humans, the role of the lung epithelium, either in the clearance of *A. fumigatus* or as a primary site of infection, is not well defined. Lung surfactant, which has to be transversed before the fungus comes in contact with the resident alveolar cells, plays a protective role against pathogens as well. It was shown recently that the hydrophilic surfactant proteins A and D enhanced agglutination, phagocytosis, and killing of conidia of *A. fumigatus* by alveolar macrophages and neutrophils (391).

An area of research which has not been explored to date is the role of damage to the lung epithelium following immunosuppressive therapies (irradiation and drugs) and GVH disease (118, 154), since it is known that binding of conidia to epithelial cells is facilitated by altered or activated epithelial cells (76). Moreover, the role of immunosuppressive drugs in facilitating the crossing of this anatomical barrier by the organism has not been studied (56, 261, 510).

Humoral components. There are few studies that address the interaction between serum components and *Aspergillus* spp. The level of fibrinogen in serum increases during the course of IA, and this substance can bind *A. fumigatus* (86, 663), but its role in invasion is unknown. C-reactive protein, an acute-phase protein whose role would be to activate the complement cascade (66, 539), can bind to *Aspergillus* fractions. The role of complement activation in protection against *Aspergillus* and other opportunistic fungal infections has been reviewed recently (316). Direct activation of the alternative pathway and binding of C3 to the fungal surface have been demonstrated (317, 546, 627). Resting conidia, swollen conidia and hyphae seem to differ in the mechanisms by which they initiate the complement cascade. Initiation is slow with resting conidia, and it has been suggested that the ability of C3 to associate is dependent on changes in the physicochemical composition of the surface of each morphological form (316, 666). Paradoxically, serum which had been heated prior to opsonization significantly increased the ability of pulmonary macrophages to kill conidia of *A. fumigatus* (547).

The production of a specific lipophilic inhibitor of the alternative complement pathway has been reported as well (711, 713). However, the nature of the inhibitor and its specific site of action are unknown. In addition, proteolytic cleavage of C3 bound to conidia by molecules present in the outer wall has been reported (626). The significance of these factors in the pathogenesis of aspergillosis is unknown. However, complement does play a role in the pathogenesis of aspergillosis, since C5-deficient DBA/2N mice are more susceptible to experimental infections than are C5-sufficient mice (94, 255). Generally speaking, activation of the alternative complement pathway favors efficient binding and fungal killing by phagocytes, but more detailed studies are needed to understand the specific role of complement during the inflammatory response to *A. fumigatus* and the initiation of activation and binding of complement by each morphological form of pathogenic and saprophytic species of the aspergilli (260).

Phagocytic cells. The role of phagocytic cells in protection against *A. fumigatus* has been studied by several research groups (562, 625). Assay conditions and cellular models vary significantly, and the results do not always agree, but in general, both in vitro and in vivo studies demonstrate a major role for phagocytic cells in protection against *A. fumigatus*. The

lungs are the site of infection of *Aspergillus* sp., and since alveolar macrophages are the major resident cells of the lung alveoli, they, along with neutrophils (which are actively recruited during inflammation and may outnumber macrophages), are the major cells involved in the phagocytosis of *A. fumigatus* (Fig. 6). Descriptions of the interaction of each host cell with *Aspergillus* is presented below.

(i) Macrophages. The recognition, binding, and ingestion of conidia by alveolar macrophages have been poorly studied, although lectin-like interactions are thought to be primarily responsible for the adherence and ingestion of conidia (297, 298). This interaction would be expected since the alveolar environment of the resident macrophage is probably free of opsonic factors such as complement and Igs. The mannosyl-fucosyl receptor and two other receptors (inhibited by glucan and chitooligosaccharides) have been suggested to mediate conidial binding, but specific receptors have not been identified (297, 298).

As a result of the attachment and ingestion of conidia, a typical phagocytic response occurs (544, 547, 698, 714). Killing of conidia starts after a delay of several hours, with a surprisingly low killing rate of 90% in 24 h (587, 589, 592). These data correlate well with the slow elimination of conidia from the lungs of mice following a respiratory challenge. Additionally, the competence of macrophages to kill conidia depends on the anatomical source of the phagocyte: alveolar and blood-derived macrophages from mice, rabbits, and humans prevent conidia from germinating, whereas resident peritoneal macrophages from the same animals do not (592). The antimicrobial system(s) responsible for killing conidia has not been identified, but most data suggest that reactive oxygen intermediates do not play a role in the killing of *A. fumigatus* conidia by macrophages (163, 417, 559, 564, 587, 588, 592, 645).

Several lines of evidence suggest that nonoxidative mechanisms are essential for the killing of conidia: (i) conidia triggered a respiratory burst when they were ingested but were killed hours later when oxidative killing systems were no longer operative; (ii) conidia were killed by rabbit alveolar macrophages under strictly anaerobic conditions; (iii) human blood macrophages cultured in vitro for 10 days were still able to kill conidia although they had lost their capacity to produce significant amounts of reactive oxygen intermediates; and (iv) monocytes from patients with CGD and from X-CGD mice, which have a genetic defect in the phagocytic burst, killed conidia as well as control, normal phagocytes did (432). The role of nitric oxide in the killing of *A. fumigatus* conidia has been insufficiently investigated (417, 645). The fungicidal activity of alveolar macrophages was unaltered in the presence of the competitive inhibitor *N*-monomethyl-L-arginine, suggesting that nitric oxid was not involved in the killing capacity of murine and human macrophages. Cationic proteins and antifungal enzymes have potent antifungal activity (162, 163). Macrophage proteolytic activity is induced significantly by *A. fumigatus* antigens (553).

Killing of phagocytosed conidia was delayed until the conidium had swollen. Intracellular trafficking of phagocytosed conidia has been poorly studied (411, 412, 457). Killing of conidia is followed after 6 to 12 h by complete digestion of conidia. The in vitro sensitivity of swollen conidia to macrophage killing is higher than that of resting conidia, but it may be due to the oxidative burst which followed the ingestion of swollen conidia (362, 366). A 100% killing of inhaled conidia by alveolar macrophages has never been reported. Furthermore, conidia can germinate in monocytes (591, 592, 712), suggesting an essential role for the second line of phagocytic cells, the neutrophils, in containing the conidia that resist intracellular killing or are not ingested before germination.

(ii) Neutrophils. PMN were thought to act exclusively on hyphae, as opposed to conidia, of *A. fumigatus* (591). However, several studies have shown that they are also able to ingest and kill resting or swollen conidia not previously killed by macrophages (191, 359, 363, 365, 479, 627, 697). Nevertheless, neutrophils remain responsible primarily for hyphal, not conidial, killing. Neutrophils adhere to the surface of the hyphae, since hyphae are too large to be engulfed, but the process of adhesion has been poorly studied. It is known that even though complement and antibodies bind avidly to hyphae, their presence is not required for the interaction between hyphae and neutrophils (627). Fungal and PMN receptors involved in this interaction remain to be identified.

Contact between neutrophils and hyphae triggers a respiratory burst, secretion of reactive oxygen intermediates, and degranulation (162, 284, 364, 365, 413, 590, 591). In contrast to the killing of conidia by macrophages, hyphal damage by PMN was rapid, in that 50% of the hyphae were killed after a 2-h incubation (565). Killing of hyphae required oxidants, but PMN oxidant release could not mediate hyphal killing without concomitant fungal damage by granule constituents (162, 366). Electron microscopic observations have shown that PMN-induced cell wall damage was detectable before killing occurred and that incubation of hyphae with granules isolated from PMN resulted in a very rapid release of cell wall glycoproteins (<30 min) (164, 527). The enzymes responsible for cell wall injuries have not been identified. However, polysaccharide hydrolases, which have been isolated from phagocytes, may play an essential role in this process (2, 187, 477, 478).

It is clear that the mechanisms responsible for killing have not been fully identified. Experiments with cells from patients with CGD or myeloperoxidase deficiencies (162, 163, 526, 527) have shown that at least two oxidative pathways are involved, but the target biomolecules of oxidant-mediated damage (lipid peroxidation, protein oxidation, or DNA degradation) are unknown. As well as oxidative mechanisms, nonoxidative killing mechanisms, such as the defensins, may be active against hyphae and germinating conidia (366, 712).

(iii) Platelets. In humans, platelets play a role in protection against *Aspergillus* (103). The importance of intravascular defenses against *A. fumigatus* is usually underscored by the extensive hyphal invasion of blood vessels, leading to thrombosis and hemorrhagic infarction. Platelets attach to cell walls of the invasive hyphal form of *A. fumigatus* and become activated during attachment to hyphae. Optimal activation requires opsonization of hyphae with fresh or heat-inactivated whole plasma. Several anti-*Aspergillus* functions, including direct cell wall damage and enhancement of neutrophil-mediated fungicidal effects, have been associated with platelets. As a possible consequence of the role of platelets, thrombocytopenia, which has been associated with prolonged neutropenia during chemotherapy, may increase the risk for these patients to become infected by *A. fumigatus*.

Acquired Immunity

T-cell immunity. Cell-mediated immune responses and type 1/type 2 cytokine dysregulation has been studied extensively in human mycosis (388, 449). A type 1 response, which is usually associated with a strong cellular immune response and increased levels of IL-2, gamma interferon (IFN-γ), and IL-12, favors resistance to mycotic disease. A type 2 response is usually associated with a minimal cellular response and an increase in antibody production, with the associated production

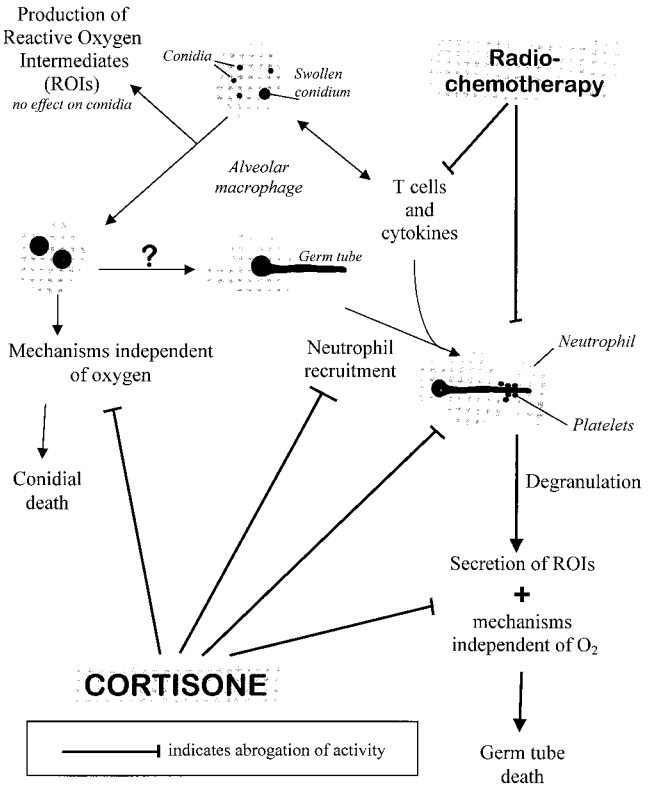


FIG. 6. Role of the host innate immunity against *A. fumigatus* and its modulation by immunosuppressive agents used in BMT.

of IL-4, IL-5, and IL-10. This pattern of reactivity can be associated with pathological findings as well. Nevertheless, until recently, few studies have focused on acquired immunity to *Aspergillus* infection. These studies have been concentrated primarily on two types of experimental murine models of aspergillosis: ABPA following inhalation of *A. fumigatus* antigens and IA following i.v. injections of conidia. Although it has become evident that the Th1-Th2 pattern of T cell response is too simplistic in that many cytokine patterns cannot fit perfectly into a Th1, Th2, or Th0 classification (600), this dual presentation is used to explain the conclusions of the studies on T-cell immunity during aspergillosis (Fig. 7).

In mice, as in humans, ABPA is associated with a pulmonary eosinophilia, a Th2 cytokine profile manifested by the production of IL-4, IL-5, and IL-10, and an increase in levels of total and specific IgE, IgG1, and IgA, reflecting the Th2 humoral response to *A. fumigatus* antigens (106, 107, 310, 326, 330, 332, 340, 707). An increase in the productions of the proinflammatory cytokines, IL-1α and tumor necrosis factor (TNF-α), associated with an upregulation of intercellular cell adhesion molecule 1 (ICAM-1), was seen in the early stages of murine ABPA (108). The Th2 cytokine pattern is seen either directly through histological staining or indirectly from isolated, activated lung lymphocytes (106, 326, 340, 447). The use of anticytokine MAbs and transgenic mice has confirmed the involvement of the Th2 pattern in ABPA. Anti-IL-4 antibody treatment resulted in a suppressed Th2 response and an enhanced Th1 response. Similarly, in IL-4 knockout mice, a Th1-type response was seen, with no demonstrable IgE and high levels of IFN-γ-dependent IgG2a (330, 331). Anti-IL-5 antibody abrogated eosinophilia in mice, whereas those treated with anti-IL-4 antibody had reduced IgE levels comparable to those in controls (337, 340, 446). Thus, IgE and eosinophilia are independently regulated. The role of IgE and eosinophil recruitment in the pathological findings of ABPA remains controversial, since IgE-deficient mice and B-cell-deficient mice, as well as IL5-deficient mice and mice treated with anti-IL-5 antibodies, still develop histological lesions similar to those found in control mice (120, 330, 404). Moreover, the route of inoculation, the type of antigen, the presentation of the antigen, and the strain of mouse are essential in determining the immune response (326–328). The use of particulate antigens has suggested the occurrence of a localized Th2 response in the organ directly exposed to the antigen. The overall immunostimulatory effect of particulate antigens seems more pronounced than that of soluble antigens administered i.n.; likewise, these antigens enhance the Th2 pattern. *Aspergillus*-specific $CD4^+$-T-cell clones isolated from ABPA patients have a Th2 phenotype (97, 310). In humans, the increased Th2 response is associated with a decreased or suppressed Th1 response (310, 330). Genotype analysis of the T-cell clones isolated from ABPA patients and specific for the dominant ASPF1 antigen are mostly restricted by HLA-DR molecules (97, 98, 310). Serotyping revealed that 90% of the ABPA patients were either HLA-DR2 or HLA-DR5 or both, a proportion significantly higher than in a normal control population. In contrast, healthy donors respond with a Th1-type response to *A. fumigatus* conidia with increased levels of IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α (225).

Studies of cell-mediated immunity to IA have been few, although the experimental systems are available today to assess the role of the different T-cell subsets and their associated cytokines in protection (699); these include (i) mouse strains that have different susceptibilities to *A. fumigatus* infections (DBA2 mice are more susceptible than BALB/c, C57BL/6, or CD2F1 strains) (94, 255), (ii) murine models in which healing and nonhealing patterns of infection can be obtained following a primary inoculation with a nonlethal concentration of conidia (94, 157, 357), and (iii) mutants of *A. fumigatus* with different growth characteristics and different levels of virulence (24, 139, 283, 408, 437). By using i.v.-challenged immunocompetent mice as well as cyclophosphamide-treated mice infected i.n., it has been shown that progression of the disease is associated with a type 2 response correlated with an increase in IL-4 and IL-10 levels and a lower level of IFN-γ (94). In contrast, development of protective immunity is associated with the presence of $CD4^+$ T cells producing IFN-γ and macrophages producing IL-12. However, administration of recombinant IL-12 (rIL-12) failed to increase resistance in nonhealing mice, confirming the inability of rIL-12 to exert protective effects in murine models of invasive fungal infection. Administration of IFN-γ, TNF-α, or soluble IL-4 receptor, on the other hand, rescued infected animals. In contrast, the early control of infection is greatly impaired in TNF/$LT\alpha^-$ deficient mice (93, 452), and IFN-γ neutralization resulted in increased pathological findings and a concomitantly increased expression of IL-10 mRNA. The IL-5 level was not increased in mice susceptible to IA. In IA, as in ABPA, the production of IL-5 and that of IL-4 and IL-10 are differently regulated (93). The antibody pattern has been insufficiently studied in these models and has shown only that IgE levels were increased in both types of mice following infection, although to a lesser extent in leukopenic mice (93). In addition, in contrast to ABPA, the increase in IgE concentration which follows Th2 activation did not follow the increase in IL-4 levels during infection in a model of IA (94).

The role of IL-10, a typical Th2 regulatory cytokine, in

aspergillosis is controversial, although neutralization of IL-10 increases the resistance of cyclophosphamide-treated mice to IA (93, 560). Accordingly, IL-10-pretreated human macrophages exhibited suppression of O_2^- production and decreased hyphal damage. Surprisingly, phagocytosis of conidia and intracellular killing was enhanced by IL-10 pretreatment. IL-4 did not alter the upregulatory effect of IL-10 on phagocytosis. These results are puzzling and may suggest that the same cytokine, in this case IL-10, plays different roles at different stages of disease, a suppressive effect during late stages of the disease when hyphae predominate and an enhancing effect during an early stage when conidia predominate. A dual role for a particular cytokine would also fit the observations that have been made with ABPA, where IL-10 may be beneficial in restricting the inflammation induced by inhalation of *A. fumigatus* antigens (233). These studies clearly show that in murine models of *A. fumigatus* infections, the production of Th cytokines occurs differently in mice resistant or susceptible to infection. Studies of T-cell immunity in humans are lacking, and the putative role of these cells is suggested only by the increased incidence of IA seen in human immunodeficiency virus-infected patients, in whom the dysfunction of $CD4^+$ T lymphocytes is well known. Studies of T-cell subsets, epitope mapping, and HLA restriction should be undertaken for IA patients as well, since they may give insights into possible links between the susceptibility or immunopathological response of patients and their genetic backgrounds, an area which has been essentially ignored in nosocomial IA infections.

Protective immunity. It is possible to immunize animals with a sublethal dose of conidia inoculated i.v. or i.n. or with a killed-germling preparation given subcutaneously (94, 157, 537, 612) and to demonstrate increased resistance to a lethal challenge with *A. fumigatus*. Although studies mentioned above have shown a central role for T lymphocytes in protection, the effector cells and mechanisms responsible for this protective immunity remain unclear. In one study (94), PMNs were thought to be responsible for the killing of *A. fumigatus* in the protected mice, but in another, the role of PMNs was discarded and acquired immunity was suggested to be mediated by activated macrophages (157). Sensitization of PMNs to *A. fumigatus* in asthmatic patients or preexposure to chemoattractants stimulated phagocytosis (538, 545).

The role of antibodies in protective immunity has not been investigated properly. Transfer to naive animals of serum containing anti-*A. fumigatus* antibodies from immunized animals offered no protection, suggesting that antibodies did not play any role in protection (157). However, it was shown recently that mice immunized with conidia mounted a monospecific polyclonal antibody response against DPPV, one of the two major antigens recognized by antibodies from sera of patients with aspergilloma. The isotype of the antibody response is first IgG1 and second IgG2 and IgA but not IgG3 (346), a pattern recognized as conferring antibody-mediated protective immunity against fungal infections (92) and, paradoxically, typical of a Th2 response. Patients who survive IA also show an antibody response against specific antigens (83, 402). However, until MAbs directed specifically against putative protective antigens such as DPPV are assessed for their protective effect, no conclusions can be drawn on the role of antibodies in acquired immunity. Such studies are complicated and difficult to interpret, since autoimmunity against human homologs of *A. fumigatus* antigens such as HSP 90 (7) dipeptidylpeptidase IV or CD26 (42), and Mn-superoxide dismutase (SOD) (125) may occur. For example, humoral and cell-mediated autoimmune responses to human Mn SOD were demonstrated in vitro and in vivo in patients allergic to *A. fumigatus* (125). Future investigations should be directed toward the study of acquired immunity, since the protection of steroid-treated animals obtained suggests that an immunotherapeutic approach may be possible for immunosuppressed patients at greatest risk for IA.

Role of Immunosuppression in the Development of Invasive Aspergillosis

Immunosuppressive drugs. It has been known for a long time that down-regulation of the immune system as a result of immunosuppressive drugs triggers the development of invasive aspergillosis in experimental animal models as well as in naturally occurring human infections (61, 93, 579, 668, 724). The alterations of the anti-*A. fumigatus* phagocytic capacity of phagocytes following radio- and chemotherapy have not been characterized (117, 620). Among the drugs used in the management of patients at risk for IA, corticosteroids play a major role in disease progression (13, 48, 161, 234, 483, 532, 533, 586, 587, 594). In spite of this knowledge, the specific role which corticoids play in the interaction of *A. fumigatus* with the immune system is poorly understood.

Schaffner and collaborators have stressed that the primary role for glucocorticoids is to impair the anticonidial activity of macrophages (589). Macrophages exposed to pharmacological concentrations of glucocorticoids ingest conidia at a normal rate and respond adequately with a respiratory burst and secretion of ROIs but fail to inhibit conidium germination and to kill conidia (406, 588). This impairment is mediated by the glucocorticoid receptor, since activity can be inhibited by glucocorticoid antagonists. However, other studies have shown that treatment of elutriated human macrophages with dexamethasone suppressed O_2^- release, although, as in the previous study, such treatment significantly suppressed fungal damage caused by macrophages (559). Based on electron microscopy studies, although no specific lysosomal staining techniques were used, it appeared that glucocorticoids stabilized lysosome membranes and prevented phagolysosomal fusion during *A. fumigatus* phagocytosis (411, 412); however, data from other studies with lysosomal markers such as acid phosphatase or acridine orange have not confirmed this hypothesis (586, 594). Activation of macrophages by IFN-γ or TNF-α did not abrogate the functional impairment of the nonoxidative killing systems caused by glucocorticoids despite an augmentation of the oxidative killing capacity by lymphokines, as evidenced by antibacterial activity, in the presence of pharmacological concentrations of glucocorticoids (406, 589, 593). In contrast, Roilides et al. (559, 561, 564) have shown that M-CSF, GM-CSF, and IFNγ prevented dexamethasone-induced immunosuppression of anti-*A. fumigatus* monocyte activity and restored O_2^- production and hyphal damage to control levels. The nature of the anticonidial, nonoxidative killing mechanisms of macrophages which are abolished by glucocorticoids remains unknown.

Pharmacological concentrations of corticosteroids are also known to suppress infiltration by PMNs as well as selected functions thereof (29). Hydrocortisone succinate and dexamethasone suppressed PMN O_2^- production in response to both opsonized and nonopsonized hyphae. Both molecules suppressed PMN-induced hyphal damage at 10 to 1000 μM, depending on the drug used (566), confirming the role of reactive oxidants in the killing of hyphae. However, it is worth noting that the concentrations of glucocorticoids used were higher than pharmacological concentrations (1 and 30 μM for dexamethasone and hydrocortisone succinate, respectively). Indeed, macrophages seem more sensitive than PMNs to glucocorticoids, since the dexamethasone concentrations used to

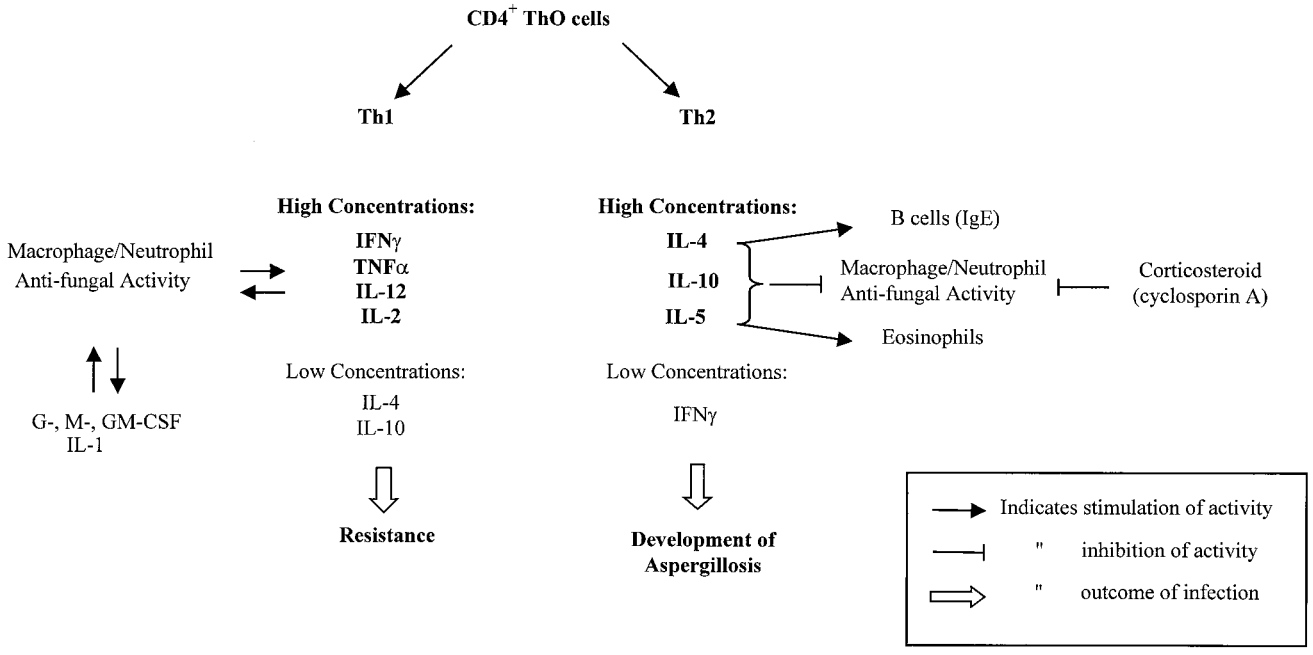


FIG. 7. Cytokines and their possible role in modulating cellular immunity in response to *A. fumigatus* in experimental murine aspergillosis.

inhibit PMN were 100-fold higher than the concentrations which induced anticonidial activity of macrophages (10^{-7} to 10^{-9} M) (559, 560). The glucocorticoid-induced suppression of PMN antifungal activity could be rescued by the addition of G-CSF and IFN-γ, which restored O_2^- production (565). The rapid suppression of O_2^- production (30 min) suggests, similar to the conclusions for macrophages, a direct down-regulatory action of glucocorticoids on specific surface receptors of PMN.

In contrast to cortisone, therapeutic concentrations of cyclosporin A do not suppress antifungal activity of PMNs and alveolar macrophages (563). However, cyclosporin A may induce significant immunosuppression of phagocyte antifungal function at relatively higher concentration in vitro, especially when combined with corticosteroids.

The role of immunosuppressive drugs in the T-cell response to *A. fumigatus* has not been investigated carefully. Cyclophosphamide-treated mice develop a Th2 response in a way similar to immunocompetent mice upon *A. fumigatus* infection (93). It is not known if the reduced conidial and hyphal killing seen in these animals is due to the direct effect of the drug or to a secondary effect on T cells or both. Although cortisone is also known to block cytokine activity, its effect on cytokine production has not been investigated in a pathological situation. Schaffner et al. (587, 591) do not believe that T-cell-mediated immunity is the target for cortisone, since the drug affected athymic mice in a manner comparable to its effect on normal mice. Dexamethasone has a much larger direct effect on the anticonidial activity than IL-4 and IL-10 do (406). In the ABPA model, cortisone inhibits pulmonary eosinophilia and IgE levels whereas cyclosporin A upregulates these functions (705, 706). Upon injection, dexamethasone significantly inhibits an *A. fumigatus*-induced increased number of activated T cells and cytokine-expressing cells in parallel with a decrease in pulmonary eosinophils and in the levels of IL-4, IL-5 and GM-CSF. Interestingly, both cortisone and cyclosporin A inhibit the production of anti-*A. fumigatus*-specific IgE and IgM, but the antibodies which are inhibited have different specificities, as shown by Western blot analysis (705). Obviously, more work should be done on the role of glucocorticoids in the regulation of T-cell and phagocyte responses against *A. fumigatus*.

Cortisone has also been proposed to act directly on *A. fumigatus*, enhance the metabolic activity of hyphae, and stimulate moderate growth (460, 565). Our results, however, suggest that the addition of cortisone to axenic media did not significantly enhance the mycelial growth rate of the strains tested (582), although some steroid hormones are known to be catabolized by *A. fumigatus* (615). These results suggest that the glucocorticoid target is phagocytic cells, not the fungus.

Immunosuppressive molecules of fungal origin. In addition to the immunosuppressive role of glucocorticoids, *A. fumigatus* secretes molecules which are potentially immunosuppressive. The role of toxic secondary metabolites in perturbing the phagocytic defense response, as well as the mucociliary activity, is mentioned above. Other low molecular weight molecules secreted by *A. fumigatus* conidia are also able to affect both phagocytosis and the release of superoxide anion and hydrogen peroxide (419, 448, 544, 548–550, 610). One of these inhibitors was heat stable and secreted prior to germination, within minutes after conidia were suspended in the cell culture medium (548–550). The diffusible compound(s) acted similarly to cytochalasin B and reduced the ability of phagocytes to migrate due to its interaction with the contractile elements of the cell cytoplasm. It also inhibited the production of IFN-γ and IL-6 at the level of transcription. The loss of cytokine gene expression in the macrophage resulted from the blocking of the transcription factors NF-κB and AP1 by the conidial inhibitor (462). This molecule has not been chemically identified but appears to be different from gliotoxin, which has similar effects on transcription factors (482).

MOLECULAR EPIDEMIOLOGY AND PROPHYLAXIS OF INVASIVE ASPERGILLOGIS

As mentioned above, soil is the natural ecological niche of *A. fumigatus* from which aerosols of conidia are released. Thus, the pathogenesis of IA starts with the inhalation of conidia by

the patient. Any increase in the concentration of airborne conidia increases the risk of contracting aspergillosis in susceptible individuals (4, 114, 199, 530, 556, 703, 716). Indeed, hospital outbreaks of IA in the 1980s were frequently associated with renovation and construction of buildings and, probably, the resulting short-lived, concentrated burst of airborne *Aspergillus* (5, 17, 18, 160, 387, 465, 466, 597, 604, 616, 624). As a consequence, strict prophylactic measures have been introduced which have resulted in a reduction of aerial contamination in the hospital setting. The use of laminar flow in selected hospital rooms has made it difficult to isolate *A. fumigatus* from these rooms, although in other sectors of the hospital conidia are always present at low levels throughout the year, with a range of concentrations between 0 and 50 conidia/m^3 (99). No seasonal or daily cycle or specific activities in the hospital seem to be associated with the concentration of conidia inside the hospital (99, 222, 271).

The most common method of measuring airborne conidial loads is an air filtration device (306, 342). Other methods estimate the level of contamination by determining the number of conidia adhering to the walls of the patient's room by using contact methods with petri dishes or swabbing with cotton-coated applicator sticks. The latter method measures contamination which occurred at some time in the past, since conidia will settle and remain on surfaces, whereas air sampling gives an indication of the contamination in the air at the time the sample was taken. Each sampling method has its merits, and at present no method is standardized. Moreover, there is no reasonable estimation of a threshold conidial concentration above which the risk for IA increases. Additional research should be done in this area, with sequential air sampling being performed to establish statistically a potential correlation between the number of conidia in the air and the risk for IA.

Chemoprophylaxis has not been shown conclusively to be efficient in reducing the incidence of IA. A few randomized trials have indicated that i.n. sprays of AmB can reduce pulmonary colonization by the organism (51, 52, 109, 115, 131, 262, 288, 659). However, new multicenter studies are needed to evaluate the role of the chemoprophylaxis (172, 414). A dose of AmB at 1 mg/kg/day has proven to be of some benefit, but the efficacy of low doses (0.1 to 0.5 mg/kg/day) remains debatable (140, 541, 570). To date, the only prophylactic method which appears efficient is the reduction of conidial contamination of the air in specific hospital settings, especially those in which neutropenic patients are housed (31, 53, 281, 282, 604, 703). The Centers for Disease Control and Prevention (Atlanta, Ga.) recommends the use of sealed rooms capable of achieving at least 15 air changes/h, with air being filtered by high-efficiency particulate filters (HEPA) with >95% efficacy for removal of 0.3-μm-diameter particles, the maintenance of positive pressure relative to the hallway, and the use of directed air flow within the room (58, 199, 475, 529, 703). The use of HEPA filters has significantly reduced the number of IA cases. Laminar-air-flow rooms seem to protect against early infections, but numerous IA cases continue to occur, suggesting that patients have not been sufficiently isolated or that colonization of the lungs occurred before isolation of the patient. Complicating the situation, however, is that fact that most IA infections occur >2 months after grafting, when the patients are being monitored as outpatients, casting doubt on the ability of laminar-air-flow rooms to prevent IA (692).

The lack of understanding of the microenvironmental niche of the organism has resulted in the supplementation of classical epidemiologic studies with genomic typing data to investigate the specific source of *A. fumigatus* infections, with the goal of being able to eradicate the fungus from that environment. The repeated DNA sequence (λ3.9) mentioned above (459) is the only probe currently available that has provided an efficient and precise computer-based analysis of DNA fingerprints of large populations of *A. fumigatus* strains (133). By using this probe, *A. fumigatus* populations were monitored in different hospital settings over a long period, up to 2 years, to determine the variability and/or persistence of the airborne *A. fumigatus* population over time (99). The analysis was extended to include about 2,000 isolates collected from around the world, so as to investigate the biodiversity of clinical and environmental isolates of this fungal species (133).

The results of fingerprinting clinical isolates from multiple sites in a given patient with aspergilloma or IA indicate that most of the patients were infected by a single strain (99, 133, 219, 220). However, in a selected proportion of patients, tested under conditions which excluded the possibility of accidental contamination of biological samples, two strains were isolated, suggesting that mixed infections with unique strains of *A. fumigatus* can occur in IA. Although most patients with IA were infected with a single genotype, 5 to 10 different genotypes per patient were isolated from cystic fibrosis patients without ABPA (458). The same genotypes were found repeatedly over time, however, demonstrating for the first time that cystic fibrosis patients are indeed colonized permanently by the same strains of *A. fumigatus* but that the strains do not cause significant pathological changes. This continuous colonization explains the rise of anti-*Aspergillus* antibody levels often seen in these patients in the absence of clinically apparent aspergillosis.

Genetic analysis cannot discriminate between clinical and environmental isolates of *A. fumigatus* (133), indicating that every strain present in the environment is a potential pathogen if it encounters the appropriate host. This observation has practical implications, since it indicates that preventive measures should be applied to all environmental sources of *A. fumigatus* conidia. The absence of environmental strains of *A. fumigatus* with reduced virulence is in agreement with previous biochemical and molecular as well as immunological studies, which have proven incapable of identifying a key factor responsible for the pathogenicity of *A. fumigatus*. The ability of any strain of *A. fumigatus* to become pathogenic in contact with an appropriate host also provides evidence for the absence of host specificity. For example, host-specific strains were not observed when isolates from cows and humans were compared (133).

The extreme biodiversity of this fungus is also seen when the geographical origin of the strain is considered (99, 133). Similar mean percentages of relatedness, all low, were found when strains (i) from two hospitals in Paris, (ii) from one hospital in Paris and a random selection of strains from the rest of the world, or (iii) from Europe and North America were compared. Thus, no clustering of strains which could be attributed to geographic location was observed.

In a confined area such as a hospital, airborne conidia are extremely diverse, with 85% of the strains being isolated only once and each displaying a unique fingerprinting pattern. The remaining 15% of the strains, accounting for >30% of the isolates, were each isolated on several occasions, and each could persist for several months in the same hospital environment. No particular strains were isolated repeatedly in the same specific location within the hospital, but identical strains were isolated from different buildings of the hospital and its environs. Common strains were found, although at a low level, in two Paris hospitals. On balance, these results suggest that the majority of strains isolated are specific to each hospital and originate less commonly from the local outside environment.

The isolation of an identical strain from a patient and from

the hospital environment of that patient indicates that the infection was nosocomially acquired (81, 99, 220, 354, 642). In a study involving isolates from 77 patients at different hospitals, however, identical isolates were collected from 2 patients or from a patient and his or her hospital environment only 39% of the time (99). Using this criterion, about 40% of IA cases appear to have a nosocomial origin. However, even during outbreaks of aspergillosis, multiple patients are rarely infected by the same strain (99). Data involving typing of environmental isolates indicated that each patient was surrounded by an extremely diverse population of strains. Therefore, two patients would not be expected to necessarily develop disease as a result of infection by a single genotype. For example, assuming that a patient stays in an environment with 1 conidium/m^3 of air for 3 months after BMT and before developing IA, that patient would have inhaled about 8,000 different genotypes during this period (99). The absence of identity between genotypes found in patients and those found in their environments should not exclude a nosocomial origin for an infection. It only indicates that the environmental population of conidia typed reflected a partial sampling of the multiple genotypes inhaled by each patient. Moreover, the nosocomial nature of IA can be demonstrated even after several weeks of delay between acquisition of the fungus and development of IA. Strain identification must be checked carefully before IA infection can be designated as community acquired rather than nosocomial (491).

The question of the origin of the variability encountered within this species remains unanswered, since no teleomorph has been found (see "Taxonomy of *A. fumigatus*" above), but three hypotheses have been promulgated. First, it has been suggested that continuous genetic exchange can occur through a parasexual cycle, so that *A. fumigatus* remains a continuously evolving species. Strains of *A. fumigatus* that are compatible with respect to their vegetative hyphae have been found (100), allowing for the possibility of the presence of a parasexual cycle in this species. However, it has never been shown that a nonmeiotic parasexual cycle can account for recombination in a natural population of any fungus. Second, it has been suggested that variability was established long ago through meiotic exchange at a time when *A. fumigatus* had a sexual stage. This hypothesis is supported by the occurrence of a relic of a RIP process in this species (459). Continuous exchange of conidia through air currents throughout the world, in addition to the absence of any adaptation to a parasitic condition, would explain the lack of recovery of identical multilocus genotypes from geographically and temporally unassociated hosts. Finally, it has been suggested that a sexual stage in *A. fumigatus* exists but remains undetected. Recent studies of population genetics have suggested that such a cryptic sexual stage may exist in other anamorphic *Aspergillus* spp. such as *A. flavus* (212). The presence of a sexual stage in *A. fumigatus* would allow genetic variations to occur through meiotic recombination.

TREATMENT OF ASPERGILLOSIS

At present, only AmB and itraconazole are available to treat aspergillosis (140–143, 154, 175, 209). In spite of their activity in vitro, the efficacy of these drugs in vivo against *A. fumigatus* remains low, and as a consequence, mortality from IA remains high. In this section the current literature on the treatment of aspergillosis is summarized.

Amphotericin B

AmB is still the "gold" standard for antifungal therapy of invasive aspergillosis (69). Although this drug has been used in the therapy of fungal infections for more than 30 years, its mode of action is not completely understood. AmB binds to membrane sterols, creating transmembrane channels which result in an increased permeability to monovalent cations (62). It also inhibits proton ATPase pumps, depleting cellular energy reserves, and promotes lipid peroxidation, resulting in a corresponding increase in membrane fragility and inducing Ca^{2+} leakage (70, 517, 631). AmB has two major drawbacks. First, because of its insolubility in water, it has to be solubilized in an aqueous milieu to become biologically active. To overcome this problem, the first AmB formulation, Fungizone (marketed by Bristol-Myers Squibb), was a mixture of AmB with the detergent deoxycholate in the ratio 3:7. The second drawback is the toxicity of the molecule to the patient (112, 490, 494). Severe side effects, particularly nephrotoxicty (synergistically promoted in presence of cyclosporin A), are common. Although AmB damages fungal cells to a greater extent than mammalian cells, the reason(s) for this selective specificity remains poorly understood. A commonly presented explanation is a preferential binding of AmB to ergosterol found in fungal membranes rather than to cholesterol found in mammalian membranes. However, no consensus hypothesis has been proposed to explain the mechanism of channel formation in fungal and mammalian membranes (69). In addition, the role played by the binding of AmB to serum lipoprotein and its subsequent internalization through low-density lipoprotein receptors in promoting toxicity has not been explored in great detail (681, 710).

MICs of AmB in vitro vary from 0.5 to 2 μg/ml depending on the formulation tested (188, 269). Resistance of strains of *A. fumigatus* to AmB, which has recently been observed, is rare (689). The daily administration of 1 mg of Fungizone per kg would be equivalent to a maximal concentration of 2.5 μg/ml in situ (140, 141, 209). In vitro and in vivo correlations are not necessarily exact, but these data, in combination with data gathered in clinical studies, have led to the following guidelines. Therapy should be initiated with the maximum tolerated dose within the first 1 to 2 days of treatment, since there is a clear dose response to AmB for neutropenic patients with IA. The target dose should be between 0.8 and 1.5 mg/kg/day i.v. for a total dosage of 1.5 to 4 g per patient over a period of at least 2 weeks. It is not usually possible to administer more than 0.8 to 1 mg/kg to patients on cyclosporin therapy or with severe renal impairments, however (140–143, 682).

Several strategies, relying primarily on modifications of the delivery system, have been used to improve the therapeutic effectiveness of AmB and at the same time reduce its toxicity. The most promising approach has involved the modification of the physical state of AmB in different AmB lipid formulations (69, 111, 121, 378, 379). A similar strategy has been used to reduce the toxicity of nystatin, another polyene with similar solubility problems, in an i.v. formulation (701). Three lipid formulations of AmB are now commercially available (263, 623). AmB-lipid complex (Abelcet; Liposome Co.) is a concentration of ribbon-like structure of a bilayered membrane formed by combining dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and AmB in the ratio 7:3:3. AmB colloidal dispersion (Amphocil; Sequus Pharmaceuticals) is composed of disk-like structures of cholesteryl sulfate complexed with AmB in an equimolar ratio. Ambisome (Nexstar), the only true liposomal AmB, consists of small unilamellar vesicles made up of hydrogenated soy phosphatidylcholine-distearyl phosphatidylglycerol-cholesterol-AmB in the propor-

tion 2:0.8:1:0.4 (69). Incorporation of AmB into the aqueous internal compartment and direct binding of AmB to the lipid bilayer, as well as modification of the conformation of AmB and the structure of the ligand itself, vary with the structure of the liposome (multilamellar vesicle, large unilamellar vesicle, ribbon-like), the presence of saturated or unsaturated phospholipids, and the temperature for phase transition (69). Moreover, the release of free AmB molecules, which is necessary for bioactivity, is understood only for Fungizone and has not been investigated with the lipid-based formulations (355, 636). In addition, liposomal formulation still makes the drug an easy target for the cells of the reticuloendothelial system. To circumvent this drawback, liposomal AmB has been coated with polyethylene glycol and a MAb which target the drug to the lung tissue (476).

The three lipid formulations commercially available have not been used in controlled trials and compared to Fungizone. The rationale for the concentrations used, usually 4 to 5 mg/kg/day, is unclear. In one study, in fact, AmBisome at 4 mg/kg/day, given to neutropenic patients, was not found to have a therapeutic advantage over 1 mg/kg/day (186). Complicating this issue is the fact that lipid formulations have been used most often in patients who have failed conventional AmB therapy. Despite that, many patients have improved following therapy with lipid formulations (370, 405, 542). All of the lipid formulations are clearly less toxic than Fungizone (308), but they are expensive, and randomized controlled studies are urgently needed to assess the overall efficacy of the newer preparations of AmB in terms of long-term survival (121).

Itraconazole

Another antifungal agent active against *Aspergillus* sp. is the triazole itraconazole (SporanoxB; Janssen). In contrast to AmB, its mode of action is well characterized (135, 675, 677, 678, 680). The free azole nitrogen competes for oxygen with the catalytic heme iron atom of cytochrome P-450 enzymes. Inhibition of the cytochrome P-450 14α-demethylase prevents the synthesis of ergosterol in fungal membranes. The lack of ergosterol alters membrane fluidity and steric relationships for selected membrane-associated enzymes and results in the alteration of the synthetic pathway and the accumulation of phospholipids and unsaturated fatty acids within the fungal cells. Hydroxyitraconazole, a bioactive metabolite of itraconazole, has antifungal activity similar to the parent compound (272). Itraconazole binds very weakly to mammalian cytochrome P-450, which reduces considerably the toxicity of the drug in humans.

Experimentally, itraconazole is as active as AmB in vitro. Its MICs are in the range of 0.25 to 0.5 μg/ml (146, 188). However, as with AmB, itraconazole has several major drawbacks. First, no i.v. preparation is available. This precludes its use in patients who cannot swallow or who have impaired bowel absorption, because significant absorption with systemic distribution must occur for itraconazole to be successful in the treatment of IA. Second, continuous monitoring of the concentration of itraconazole in serum is recommended since absorption varies substantially among patients. Itraconazole is efficacious in patients with less severe immunodeficiencies, i.e., those who are able to absorb itraconazole from the gastrointestinal tract, such as solid-organ transplant, aplastic anemia, or lymphoma patients, and patients with disturbances in cell-mediated immunity, including AIDS. It is also efficacious in patients in whom previous AmB treatment failed or was discontinued due to nephrotoxicity (141, 147, 149, 150, 174, 176, 682). Third, strains of *A. fumigatus* that are resistant to itraconazole have been identified recently (104, 148, 152). The mechanisms responsible for resistance have not been investigated in *A. fumigatus* as they have been in *Candida* (725), but resistance appears to be mediated by one of several possible mechanisms, including energy-dependent efflux mechanisms, increased expression of the sterol 14α-demethylase, and altered affinity of the enzyme for the drug (651). Finally, any drug that stimulates P-450 metabolism (rifampin, phenytoin, carbamazepine, phenobarbitone) will interact negatively with itraconazole. Considered together, these drawbacks suggest that itraconazole should be considered a second-line antifungal drug.

In acutely ill patients with IA, loading doses of 600 to 800 mg/day for 3 to 4 days, followed by 400 mg/day, are recommended. If concentrations in serum are in the range of 2 to 40 μg/ml, a good response is likely (63, 223, 237, 678). Treatment should last for several months, but the precise duration must be determined empirically, since 1 to 26 months have been suggested in published studies. Long duration of treatment is possible since itraconazole is tolerated well by patients and has the advantage of ease of use outside of the hospital.

Another entity in which itraconazole shows promise is ABPA. Patients with this syndrome who were given long-term itraconazole maintenance therapy improved clinically, and so therapy with prednisone could be discontinued. IgE levels dropped, and improvement in pulmonary function was observed (151). However, patients with aspergilloma were less responsive to itraconazole (135).

Outcome and Trends

Although antifungal therapy has improved and drugs are now used at more appropriate dosages, mortality due to IA remains very high. A review of >1,000 cases of IA treated with AmB showed that the crude mortality rate was 86, 66, and 99% for pulmonary, sinus, and cerebral aspergillosis, respectively. Mortality rates varied with the host group and within each host group (140, 141). All untreated patients and all those treated for less than 8 days died. Only 54% of those who received 14 days of therapy responded. A better response rate was noted for renal and heart transplant recipients, but worse responses were noted for liver transplant and BMT recipients and patients with AIDS. For example, the mean mortality in BMT patients was 90%. Several factors aside from organ involvement and host group appear to be important in the prognosis (682). Early initiation of antifungal therapy is critical, underscoring the need for more sensitive diagnostic methods. Recovery from neutropenia in leukemic patients and/or reduction in immunosuppressive therapy in transplant recipients are key factors as well (140–142). Few patients with persistent neutropenia and IA survive. Indeed, resolution of aspergillosis has followed neutrophil recovery in most instances. Where the underlying condition is acute leukemia or autologous BMT, favorable outcomes are associated with shorter durations of neutropenia. Allogeneic BMT patients have longer periods of neutropenia, and so their response to therapy is often poor. Patients should be treated until a neutrophil count of $>1{,}000 \times 10^6$/liter is achieved and until there is radiographic resolution of the disease; this may take weeks or months. In BMT patients with IA, mortality occurred at the same rate in patients who were neutropenic shortly after transplantation and in patients in whom the disease developed after the neutrophil count returned to normal but who experienced GVH disease induced by steroid therapy. In BMT patients who had recovered from myelosuppression, IA risk persists even after successful engraftment (555). It is important to remember that although the number of phagocytes is important for a favor-

able outcome, their capacity for phagocytosis and killing must also be adequate.

Immunotherapy in highly immunosuppressed patients may be a helpful adjuvant to conventional antifungal therapy (320, 322, 473). The objectives of immunotherapy are to increase the number of phagocytic cells, to modulate the kinetics of these cells at the site of infection, and/or to activate the phagocytes to kill the fungal cells more efficiently. A variety of immunomodulators enhance the killing of *A. fumigatus*, but most experiments have been done in vitro or in experimental animal models. In addition to their effect on the activation of the fungicidal activity of neutrophils and macrophages, most cytokines successfully shorten the duration of neutropenia and promote an increase in the numbers of circulating phagocytes in patients at risk of aspergillosis (320, 321, 509, 528, 555, 559, 561, 564–567, 669). Most interestingly, these cytokines act synergistically at pharmacologically relevant concentrations to reverse the deleterious effects of steroids and/or cyclosporin A and to counteract the suppressive effects of cytokines such as IL-10 (509, 528, 559, 561, 564–567, 669). Two immunotherapeutic strategies can be considered. First, neutralization of the $CD4^+$ Th2 lymphocytes such as IL-4 and IL-10 will enhance resistance to the disease, but this approach has been used to date only on animals and has not been explored in humans. Second, immunomodulators with a direct positive effect on the function of the phagocytes could be directly injected into the patients. The potential relevance of these concepts has just begun to be evaluated in *Aspergillus* infections, and the most relevant cytokines in this context are reviewed in the following section.

Although TNF-α stimulates phagocytosis by macrophages and slightly increases O_2^- production by these cells, the toxicity of this proinflammatory molecule has precluded its use in humans. In contrast, recombinant IFN and the CSFs are approved for clinical use. rG-CSF not only increases the production of neutrophils but also modulates their biological function (368). The oxidative response and antifungal activity of human PMNs are enhanced by a range of rG-CSF concentrations (562). Prophylactic use of rG-CSF during neutropenia and empirical therapy for febrile neutropenic patients has been very limited, but a beneficial effect of this cytokine in the prevention of fungal infections has been reported (555). A pilot study which has shown that rG-CSF enhanced leukocyte transfusions may offer a promising approach in treating cancer patients with neutropenia and documented aspergillosis (167). GM-CSF- and M-CSF-treated human macrophages exhibit enhanced conidial phagocytosis, O_2^- production, and hyphal damage (559, 561, 564, 565). The beneficial effect of M-CSF in augmenting the pulmonary host defense was shown in a neutropenic-rabbit model of IA (562). However, the only clinical trial involving humans with IA did not show a significant beneficial effect, and the antifungal activity of human monocytes from cancer patients receiving rM-CSF was not significantly changed (456, 562). rGM-CSF seems more promising, and a reduction in the IA-related mortality rate has been seen in a few clinical trials (60, 571). IFN-γ increased the oxidative metabolism of both PMNs and macrophages. IFN-γ is efficacious in nonneutropenic patients (526). In a recent prospective trial, IFN-γ prophylaxis in CGD patients significantly reduced the rate at which IA occurred (from 24 to 4%) (208). Although initial data are promising, the number of clinical trials evaluating the cytokines as therapy for *Aspergillus* infections are few, and they are most often nonrandomized and somewhat contradictory (320, 321). As an example, a recent study of the European Organization of Research and Treatment of Cancer shows that G-CSF or GM-CSF had no impact on the outcome of IA (143). Moreover, combination therapy of hematopoietic factors with antifungal agents, a protocol that seems to be synergistic in the case of candidiasis treated with fluconazole (508, 509, 622, 734), has rarely been attempted for IA. Since data on the reduction of the incidence of IA as a result of immunotherapy remains very limited, hematopoietic compounds are not in routine use.

As expected, successful intervention in IA has been associated with better management in the use of AmB and itraconazole. These drugs can now be used at more appropriate dosages, following the recent development of in vitro susceptibility tests which correlate better with in vivo outcome (471). The proposed standard protocol for susceptibility testing was adapted from the M27-P protocol established by the National Committee for Clinical Laboratory Standards (146, 148, 152, 188, 394). A conidial suspension of 10^6 conidia/ml is inoculated into RPMI 1640 medium containing glutamine alone or with glucose and buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) and then incubated for 48 h at 35°C. The MIC is defined as the highest dilution at which no growth, as determined by visual inspection, occurs.

Therapy must be viewed today as a sequential process involving several steps, using conventional AmB deoxycholate as the first-line drug and replacing it with lipid-based formulations if renal dysfunction occurs. If the patient is able to take oral therapy, has good intestinal function, and is not taking drugs that induce the metabolism of P-450 enzymes, some physicians use itraconazole as a first-line drug. If treatment fails, therapy must be changed, of course, but a favorable response to an alternative therapy is seen almost exclusively in patients with slowly progressive disease. The most common change to initiate is the removal of i.v. therapy with AmB and the institution of oral itraconazole for follow-up and maintenance therapy.

Surgery may be considered as an alternative life-saving strategy in selected patients, and the number of successful reports in the recent literature has increased (49, 128, 574, 658, 732). Most clinicians reserve lung resection for patients with persistent lung shadows who have to undergo a BMT or for those with hemoptysis or lesions in the vicinity of great vessels (86, 141, 430, 682).

Despite much work and the development of new drugs, anti-*Aspergillus* therapy remains inadequate. The overall success rate of AmB therapy for IA is 34% (141, 221, 533). In addition, most IA cases occur in spite of empirical administration of AmB in response to a fever unresponsive to antibacterial agents. This observation underscores the basic inadequacy of drugs in vivo with activity against *A. fumigatus* and emphasizes the urgent need for new antifungal agents. Several new antifungal drugs, such as the azoles voriconazole (Pfizer), UR 9825 (Uriach) and Sch 565-92 (Schering-Plough) or derivatives of echinocandins (Eli Lilly, Merck), are under development and in clinical trials, but the results of the trials have not been released (1, 36, 37, 91, 325, 398, 450, 467, 532, 689). In addition to developing new drugs, some research has focused on combinations of existing drugs that might be efficacious (110, 461). Pharmaceutical companies continue to be actively involved in the search for new anti-*Aspergillus* drugs by using new or revisited potential cellular targets. However, none of the approaches or targets listed as the most promising and fungus specific, including disruption of cell wall biosynthetic enzymes, blocking of DNA topoisomerase activity, disruption of enzymatic pathways involved in the metabolism of essential amino acids such as lysine, tryptophan, methionine, threonine, and isoleucine or of the enzymes involved in the synthesis of sphingolipids, polyamines, and proteins, have resulted in the discov-

ery of new anti-*Aspergillus* drugs to date (24, 137, 193, 213, 238, 247, 304, 408–410, 644).

CONCLUSION

Dramatic increases in the incidence of aspergillosis caused primarily by *A. fumigatus* have occurred in recent years, due primarily to an increase in the use of more drastic immunosuppressive therapies. *A. fumigatus* has become the most important airborne pathogen in developed countries, causing a significant increase in fatal pulmonary IA, a disease which was relatively unknown 20 years ago. At that time, *A. fumigatus* was associated primarily with localized and relatively benign diseases such as aspergilloma or allergic aspergillosis. While the number of IA cases worldwide is estimated to be only a few thousand per year, the disease is very serious, with an overall mortality of >50% and a mortality approaching 100% in allogeneic BMT patients. Moreover, IA tends to occur in patients undergoing expensive treatments for underlying conditions, and it is disastrous to cure or control the underlying condition only to lose the patient to a secondary infection. Moreover, the lessons learned in the management of IA should be helpful in the control of emerging filamentous fungal diseases caused by other saprophytic fungi occurring in increasing numbers in all immunocompromised patients at risk for systemic fungal infections.

In spite of the recent progress in the study of *A. fumigatus* and its associated diseases, it is clear from this review that there is much we do not know and many avenues in need of exploration before a thorough understanding of the pathogenic behavior of this fungus is known. Because so little is known about the natural history of IA, management of this disease is empirical and extremely difficult. To better understand the disease, four primary lines of investigation, i.e., diagnosis, epidemiology, therapy, and pathogenesis, must be pursued.

(i) Recent progress in diagnosis has resulted in better cooperation among physicians, radiologists, and microbiologists. However, early and specific diagnosis is vital, since most patients are treated empirically in the absence of a definitive diagnosis. Detection of fungal molecules in biological fluids is the best strategy to obtain an exact diagnosis in the absence of specific clinical signs and symptoms. A major challenge for the microbiologist will be to develop more sensitive and specific diagnostic kits, probably involving the combined use of immunologic and molecular tools.

(ii) To continue to improve our knowledge of epidemiology, a thorough quantification of airborne conidia in the hospital setting is urgently needed to statistically establish correlations relevant to the occurrence of nosocomial IA. This must be done immediately, before any further chemoprophylactic investigations.

(iii) There are some urgent issues with regard to therapy. The emergence of strains resistant to azoles dictates that resistance mechanisms be studied carefully, since most of the newer drugs are azole based. Moreover, imidazoles and triazoles are used in agricultural management as well, and the role of their widespread environmental use in promoting the appearance of resistance in the ubiquitous, saprophytic *A. fumigatus* has not been taken into account. Since neither AmB nor itraconazole is highly efficacious against the aspergilli, there is an urgent need for new antifungal agents for the treatment of IA. Finally, immunotherapy through the use of cytokines or the development of *Aspergillus* vaccines deserves continued exploration as an adjuvant to traditional therapy.

(iv) To date, no single virulence factor has been defined in this species. The lack of identification of virulence factors is in agreement with the absence of any selective pressure on this fungus to develop in a human. *A. fumigatus* does not need a human host to complete its life cycle; indeed, encountering an immunocompetent human host generally results in the death of the fungus. *A. fumigatus* is a true saprophytic fungus, which becomes "pathogenic" only in an immunosuppressed host. Little is known of the cellular and humoral defense mechanisms which are essential for the killing of *A. fumigatus* conidia and hyphae in the immunocompetent host. Thus, immunologists are urgently needed to identify the metabolic pathways of the phagocytes responsible for the killing of *A. fumigatus* in a normal host, to study the cellular interactions of T cells and phagocytes, and to determine which of these defense mechanisms are primarily abrogated by the immunosuppressive treatments used in patients at risk for IA.

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