

INFLAMMATORY CELLS AND AIRWAY DEFENSE AGAINST *ASPERGILLUS FUMIGATUS*

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Aspergillus fumigatus is known as a cause of multiple airway diseases that range from harmless saprophytic colonization (presence of the fungus in sputum of healthy individuals) to local inflammatory disease (e.g., aspergilloma, *Aspergillus* asthma, allergic bronchopulmonary aspergillosis) to life-threatening invasive disease in immunosuppressed patients (e.g., patients with AIDS or patients who have undergone cancer therapy or organ transplantation). Fungal infections, including those caused by *Aspergillus* species, have become more important in recent years. The increased incidence may be the adverse consequence of intensified medical treatment, resulting in a high and prolonged (sometimes even lifelong) state of immunosuppression of the patient, or it may follow widespread infections, such as in the case of an HIV infection. This article focuses on airway diseases caused by *A. fumigatus* in immunocompetent patients, with special emphasis on allergic bronchopulmonary aspergillosis (ABPA). Aspergillosis in immunocompetent patients is characterized by inflammatory responses of the airways with different cell types that infiltrate the airway tissue. Simultaneously, an immunologic response is mounted against fungal antigens resulting in a significant antibody response involving different immunoglobulin classes and subclasses. The role for the inflammatory event in the pathogenesis of aspergillosis, as yet, has not been completely understood. We argue in this article, however, that the inflammatory response of the airways may

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be involved in a repetitive cycle of self damage to the airway tissue, thereby decreasing the airway defense capacity against microorganisms. In this article we give a general description of the mucosal airway defense against fungal infections and discuss the interactions of *A. fumigatus* with the airway defense system that may lead to the inflammatory airway response. Finally, the rationale for the use of corticosteroids and their effects on the airway inflammation in patients with ABPA are discussed. We begin our discussion with a general description of the inflammatory responses in patients with either ABPA or aspergilloma.

INFLAMMATORY RESPONSES OF THE AIRWAYS AGAINST *A. FUMIGATUS*

Immunocompetent patients with aspergillosis may be distinguished into two different groups: those presenting with infectious disease (often involving suppurative processes) and those with immunologic or allergic disease. Patients with aspergilloma, chronic necrotizing aspergillosis, and *Aspergillus* pneumonia may be classified in the infectious group. In these manifestations of aspergillosis, *Aspergillus* has been found to grow inside preexisting cavities or within the airway tissue. The immunologic response in these cases is characterized by the production of IgG, IgM and IgA antibodies against *A. fumigatus* antigens that can be quantified by enzyme-linked immunosorbent assay (ELISA) and double immunodiffusion. Polymorphonuclear cells, as well as other phagocytes, are the main cells involved in the host defense against *A. fumigatus*, mostly induced by a T helper 1 (Th1) type immunologic response that orchestrates the inflammatory response. Exacerbations of aspergilloma may result in clinical manifestations, such as general malaise, significant weight loss, and recurrent hemoptysis, which may cause fatal exsanguinating hemorrhage. The active state of aspergilloma is most often treated with systemic antifungal therapy to induce disease remission. However, *Aspergillus* present within the pulmonary cavity may not be reached by the antifungal drugs because of poor local blood circulation. In these cases, local administration of the drug may be warranted. In addition to medical treatment, surgical resection is still a matter of debate. Early surgery is recommended before more complicated disease has developed, whereas removal of aspergilloma is advised in more severe cases only if life-threatening hemoptysis or severe pulmonary damage occurs.⁴⁷ Although antifungal treatment is successful in some patients from a clinical perspective, the damage caused to the airway tissue by the fungal growth during exacerbations of the disease is evident in many cases. Conventional tomography or CT scans of the airways may reveal cavities that have increased in size as compared with the size when the treatment was started.¹⁰¹ A subgroup of patients with aspergilloma with a genetic predisposition for developing allergy (atopics) may also show a Th2-type immunologic response, resulting in additional IgE antibody formation to *A. fumigatus* allergens. In this atopic subgroup levels of

IgG4 antibodies are also found, which are higher compared with nonatopic aspergilloma patients or patients with ABPA.⁹⁷ Indeed, some of these patients with aspergilloma may actually develop ABPA.

In the group of immunologic-allergic disease, we can differentiate between atopic and nonatopic manifestations. In the nonatopic group, patients with fibrotic airway disease, such as *Aspergillus*-induced extrinsic allergic alveolitis (hypersensitivity pneumonitis), have been described.^{27, 92} This manifestation of *Aspergillus*-induced organic dust disease is primarily found after massive inhalation of spores, with deposition at the alveolar level. Lymphocyte-mediated tissue inflammatory responses may underlie the pathogenesis of this form of hypersensitivity pneumonitis, which is immunologically characterized by IgG, IgA, and IgM antibody formation, delayed cutaneous hypersensitivity (48 hours), and positive lymphocyte transformation to *A. fumigatus* antigen.¹¹² In the atopic subgroup of patients with aspergillosis, a Th2-type immunologic response may dictate the production of IgE antibody against fungal allergens followed by an increased sensitization of mast cells. A corresponding positive skin test is regularly found. With atopic aspergillosis, we may discriminate the uncomplicated form of *Aspergillus* asthma from the more complex manifestation observed in ABPA. In the latter case, the disease is characterized by recurrent and long-lasting attacks of airway obstruction and, if it remains untreated, extensive destruction and fibrosis of the airway tissue.

ASPERGILLUS ASTHMA AND ABPA

Sensitization to fungal allergens, such as those from *Aspergillus*, generally occurs in combination with other inhalant allergens, such as house dust mites or tree or grass pollen; an isolated sensitization to fungal allergens is rare. In atopic individuals with fungal asthma, the exposure to fungal spores and mycelial fragments results in the production of IgE antibodies against the fungal allergens. The most important fungi that have been studied are *Alternaria*, *Cladosporium*, *Penicillium*, *Aspergillus*, and *Basidiomycetes*.^{6, 46, 49, 54, 56, 68, 89} Inhaled spores of these fungi that become impacted on the epithelial lining fluid are removed effectively by transportation to the larger airways and the oropharyngeal cavity. Because of the limited contact with fungal antigens in these cases, precipitins measured by double-immunodiffusion and ELISA-IgG titers are generally negative.^{6, 23, 54} Only the smaller allergens with a relatively low molecular weight that easily become soluble in watery solutions may reach the immunologic system, resulting in a Th2-type immunologic response with the formation of IgE antibodies to *A. fumigatus* allergens and sensitization of local mast cells (Fig. 1). Repeated exposure to fungal allergens may cause mast cell degranulation and eosinophil infiltration, and indeed, after allergen challenge of patients with fungal asthma, early or late asthmatic responses may result.^{58, 93} The immunologic and inflammatory response in fungal asthma, responsible for the

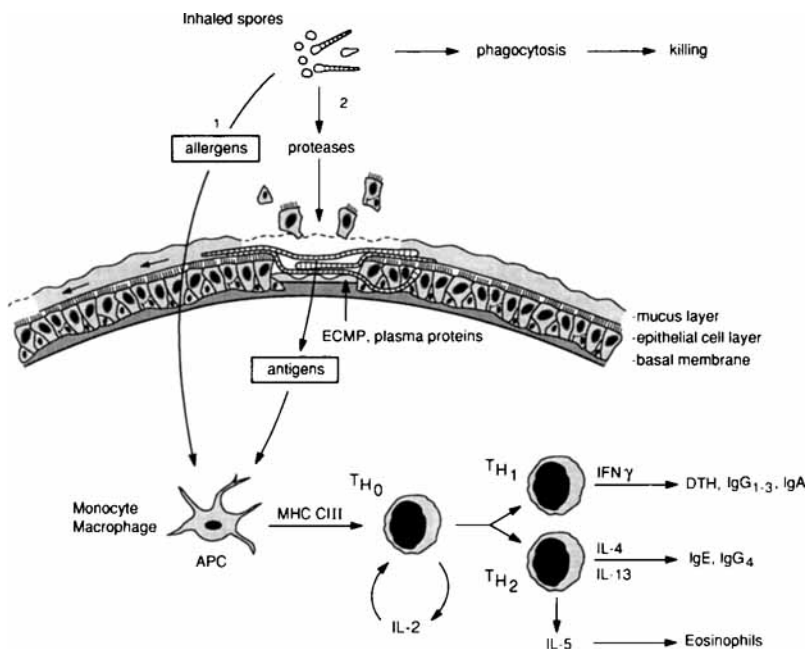


Figure 1. Defense of the airways against *A. fumigatus*—induction of the specific immune response. There are two ways for allergens to be presented to the monocyte/macrophage and lymphocyte system. Pathway 1 is passage of water-soluble allergens of relative low molecular weight that are rapidly released from the spores and may pass the epithelial cell layer in small concentrations. The atopic condition of the patient will allow the activation of the Th2-type response to low concentrations of allergens, resulting in IL-4, IL-5 and IL-13 formation, switching B cells to IgE formation against allergens, and generation of eosinophilic leukocytes. Under these conditions activation of the Th1-type response will be low or absent. Pathway 2 binds *A. fumigatus* to damaged sites of the epithelial surface, possibly induced by the release of proteases. This binding results in release of antigens and allergens in high concentrations, which activates both Th1- and Th2-type responses. As a result, IgG and IgA form, as well as extremely high concentrations of IgE antibodies against molecular components of the fungus.

fungus-induced early and late asthmatic reactions, is similar to the responses induced by other inhalant allergens, such as the house dust mite.^{36, 45}

ABPA belongs to the infectious type of asthmatic airway disease typified by growth of *Aspergillus* in the airways. The clinical picture of ABPA as described by Hinson³⁵ and others is characterized by recurrent episodes of bronchial obstruction associated with various combinations of fever, general malaise, sputum production with brownish mucus plugs, peripheral blood eosinophilia, and hemoptysis (see Varkey's article elsewhere in this issue).^{15, 42} Reactions of allergic bronchopulmonary fungus with a similar clinical presentation have occasionally been described for other fungi. The chest radiograph may show typical fleet-

ing shadows or migrating infiltrates. Bronchography and computerized tomography typically show proximal (or central) bronchiectasis, which is an important feature of this disease.⁸⁴ The introduction of the high-resolution computerized tomography (HRCT) has shown bronchiectasis in most patients with ABPA.^{6, 38} The observation that bronchiectatic lesions were also observed in some patients diagnosed as having *Aspergillus* asthma suggests that *Aspergillus* asthma may be a more destructive disease than is generally assumed.⁶⁶

The clinical state of the disease varies widely in time with episodes of severe asthma and fleeting radiologic infiltrates to periods with mild asthmatic reactions that are clinically indistinguishable from uncomplicated fungal asthma.⁴¹ The stages of ABPA may include an acute episode (stage 1), a remission phase after successful treatment (stage 2), exacerbation phases of ABPA (stage 3), a more chronic corticosteroid-resistant stage (stage 4), and a phase characterized by pulmonary fibrosis (stage 5).^{71, 84} Stages 4 and 5 may develop from the more reversible stages of the disease by repetitive asthmatic episodes and subsequent damage of the airways by activation of inflammatory cells and fibroblasts. The ongoing inflammation may result in extensive fibrosis with airway wall thickening and typical seal-ring structures around the blood vessels (Fig. 2). It is important to note that the infiltrative stage of ABPA will not always present itself with an acute asthmatic phase as we observed in one of our patients⁴⁴; however, it may start with a nonasthmatic episode, yet present all the typical features of "classical" ABPA exacerbation, including the radiologic infiltrates, peripheral and local eosinophilia,

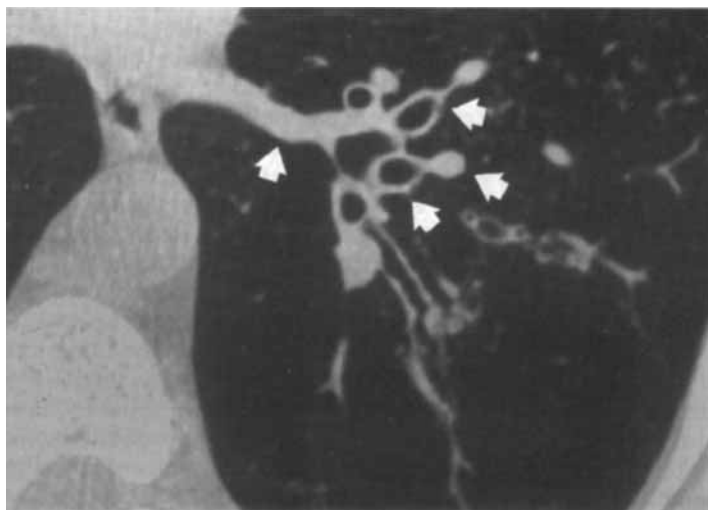


Figure 2. Severe allergic bronchopulmonary aspergillosis. Damage to the airways is shown by centrally located bronchiectatic lesions and seal-ring deformations of the vessels (arrows).

hemoptysis, and elevated immunologic responses. The nonasthmatic episode may be followed by an asthmatic episode,^{29, 85} possibly as a consequence of increased nonspecific bronchial hyperreactivity.⁴⁴

Patients with cystic fibrosis (CF) are exceptionally prone to developing ABPA. ABPA, as a complication of CF, presents with elevated IgG and IgE antibody titers, peripheral blood eosinophilia, and formation of central bronchiectatic lesions.^{37, 86, 90, 113} The subsequent damage to the airways manifests itself by a loss of airway function.^{24, 67} A more rapid decline in lung function was found in children who were sensitized for *A. fumigatus* with elevated IgE than in the nonsensitized group with normal IgE levels.¹¹¹

All manifestations of allergic fungal-induced diseases of the lower airways have also been described for the upper airways, including fungal growth in the sinuses (see Nash and Fireman's article elsewhere in this issue). In these cases, *A. fumigatus* is the most prominent fungus found causing allergic aspergillary sinusitis. Remarkably, as is the case with ABPA, allergic aspergillary sinusitis is histologically characterized by eosinophilic infiltrates.^{39, 57, 88, 103}

IMMUNOLOGIC AND CELLULAR RESPONSES IN ABPA

During exacerbations of ABPA, total IgE and specific IgE against *Aspergillus* antigens may reach very high levels, indicating ongoing allergen stimulation and an immunologic Th2-type response (see Knutsen's article elsewhere in this issue).^{43, 44} In addition to an IgE antibody response towards *Aspergillus* antigens, the humoral immune response in these patients in most cases also includes an IgE production against other inhalant allergens, indicating the atopic status of the patient.^{9, 43} During exacerbations of ABPA, the high antigen load resulting from fungal growth (Fig. 1) causes a vigorous polyclonal antibody response resulting in high IgG-, IgA-, and IgM-ELISA titers against the antigenic components released by the microorganism.^{8, 43, 54} A combination of serologic parameters may be useful in monitoring disease activity and discrimination between active, intermediate, and remission periods of the disease.^{41, 54, 71} Immunoglobulin concentrations in bronchoalveolar lavage fluid (BALF) show elevated titers of specific IgG, (s)IgA, IgM, and IgE against *A. fumigatus* that drop to normal or nondetectable levels after treatment with corticosteroids.⁴² The concentrations of proteins in BALF (e.g., albumin, transferrin, 2-macroglobulin) may also be elevated, indicating an increased transcapillary leakage because of mediators released during the inflammatory response.⁴² Local productions were observed for *Aspergillus*-specific IgA and IgE⁴² that was confirmed in other patients with ABPA.³²

Along with peripheral blood eosinophilia during exacerbations of ABPA, the study of cellular infiltrates in BALF samples and biopsy specimens have shown that the cellular infiltrates consist predominantly of eosinophils. These eosinophils may in part stain positive for activated eosinophil cationic protein (ECP) (EG2 positive), illustrating the acti-

vated state of the eosinophils.^{44, 91} In addition, epithelial damage and eosinophilic and mononuclear infiltrates have been observed in the bronchial submucosa together with a thickened collagen layer beneath the epithelial basement membrane and in the lung parenchyma.⁹¹ The inflammatory response appeared to be largely bronchocentric but may also include the alveolar level, whereas elastin layers are markedly disrupted in the bronchioles, suggesting destructive proteolytic activities at these sites.

To better understand the possible interactions between *A. fumigatus* and the airway defense system resulting in local inflammation, let us first consider some aspects of the mucosal airway defense against fungi.

MUCOSAL AIRWAY DEFENSE AGAINST MICROORGANISMS

The mucosal defense of airway of healthy individuals is a highly efficient system for the elimination of microorganisms. In the mucosal airway defense system, different functional levels may be distinguished (Fig. 3).

First the epithelial cell layer presents a physical barrier that prevents the penetration of large particles, including microorganisms. By junctional contacts the epithelial cell layer actively participates in the transport of different molecules between the epithelial cells. Excretory cells present in the epithelial cell layer produce glycoconjugates, defensive proteins, and enzymes that together constitute a protective environment.

An important function of the mucus layer (or epithelial lining fluid) of the airways is the elimination of inhaled particles, such as fungal spores, that have entered the lung and were impacted on the mucosal surface. By the coordinated ciliary movement by specialized epithelial cells, the epithelial lining fluid together with impacted material is transported out of the airways into the oropharyngeal cavity (see Fig. 3). The clearance rate of the epithelial lining fluid is regulated by the ciliary beating frequency that can be modulated by multiple factors such as drugs that activate the adenylyl cyclase system (adrenergic agents, prostaglandins), mediators that are released during infectious and asthmatic inflammatory responses, and factors excreted by microorganisms such as *Pseudomonas aeruginosa* and *Hemophilus influenzae* (for an excellent review on this subject see Wanner et al¹¹⁰). It is possible that antiasthmatic drugs that activate the adenylyl cyclase system of epithelial cells, such as inhaled long-acting beta-adrenergic agents, will give additional help to the elimination of infectious microorganisms.¹⁹

Part of the mucosal airway defense is mediated by proteins that have a direct antimicrobial activity, although the antimicrobial activity of these proteins is not dependent on the cellular defense system.³⁰ Inhibition of growth is also achieved by deprivation of essential nutrients (e.g., iron) that is strongly bound by mucosal proteins (e.g., transferrin and lactoferrin). There is increasing interest in proteins that are excreted by epithelial cells or mucosal gland cells and that possess a direct killing

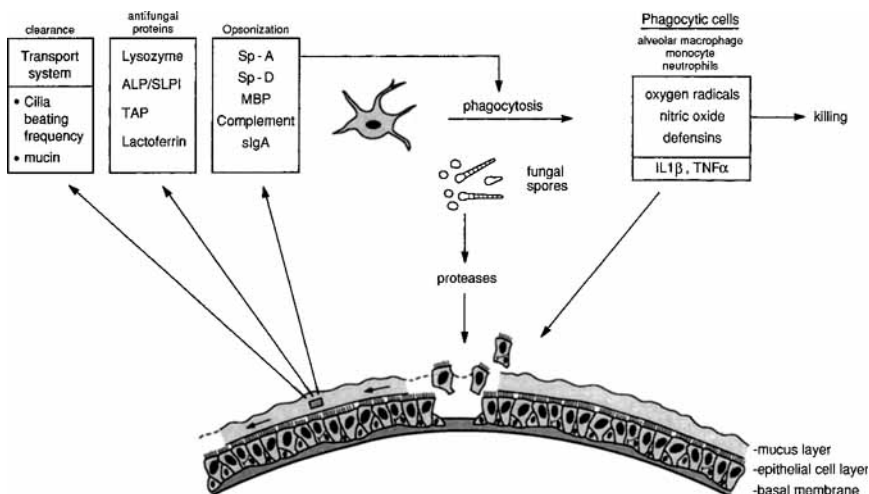


Figure 3. Defense strategies of the airway mucosa—the innate defense system. The first barrier of the airway mucosa is the epithelial cell layer bound to the basal membrane. On top of the epithelial cells, a mucus layer contains various proteins. The rate of transportation of the mucus layer by coordinated cilia beating to the oropharyngeal cavity determines the rate of clearance of particles deposited on the mucus layer. Within the mucus layer several antifungal proteins are present that cause a direct attack on the fungi, with a high-killing potency. Proteins are present that opsonize the surface of the microorganism, which will prevent binding to the epithelial layer and will help phagocytic leukocytes in the cellular killing of these fungi. By producing different proinflammatory cytokines, the epithelial cells become activated and start to produce chemokines, which will initiate an infiltration of different inflammatory cells from the blood circulation to the airways.

potential against bacteria, viruses, and fungi. In addition to the well studied enzymes, such as lysozyme, several proteins have been described that are able to inhibit the growth of or even kill various microorganisms.^{26, 30, 87} These proteins mainly belong to the family of the cationic defensins, which contribute to the innate defense in vertebrate and invertebrate organisms. These strongly cationic molecules are able to kill both bacteria and fungi. The alpha-defensins are mainly found in neutrophilic granules, whereas it was recently demonstrated that the human beta-defensin-1, excreted by epithelial cells, has bactericidal activity.³⁰ Another example is the antileukoprotease (ALP) or secretory leukoprotease inhibitor (SLPI), which has been studied with regard to its capacity to inhibit serine proteases such as neutrophil elastase.^{98, 100} Aside from being an antiprotease, strong antibacterial and antiviral activity has been demonstrated.¹⁰⁰ In a study from our laboratory, we saw that recombinant ALP (rALP) had strong antifungal capacity. rALP expressed pronounced fungicidal activity towards metabolically active *A. fumigatus* conidia and *Candida albicans* yeast cells; however, metabolically quiescent *A. fumigatus* conidia were totally resistant. The ALP molecule

is composed of two largely homologous domains with the carboxyl-terminal domain expressing the protease inhibitory activity of rALP. Remarkably, the fungicidal activity of rALP was found to be localized primarily in the amino-terminal domain. On a molar base, the fungicidal activity of rALP was comparable with the fungicidal activity of a mixture of human defensins and lysozyme. In addition, rALP caused marked inhibition of *C. albicans* yeast cell growth.⁹⁸ At the moment, there is growing evidence that more of these proteins are present in the epithelial lining fluid (ELF), which may play an important role in the defense against infectious agents such as bacteria, fungi, and viruses.^{26, 30, 87, 100}

Another level of innate defense of the airways is the function of families of proteins, such as C-type lectins, glycolipids, and glycoproteins, that are able to opsonize microorganisms by binding to surface structures.⁷² One of the important functions of these glycoproteins is to prevent the attachment of microorganisms to the epithelial surface, which is the first and most important step for a pathogenic microorganism to gain access to its host. Important components of this defense are the collectins (e.g., surfactants [SP-A, SP-D]),²¹ acute phase proteins produced by the liver (complement). The C-type lectins, complement and mannose-binding protein/lectin (MBP), act through the recognition of "patterns of molecular structures" that are specific for large groups of microorganisms.^{21, 105} In contrast to this nonadaptive defense system, secretory IgA (sIgA) is produced as the result of a specific adaptive immune response. Binding of sIgA to the surface of fungi will also prevent the binding of the spores to epithelial surfaces. Furthermore, it has been shown that sIgA complexed to immobilized antigen is important for the activation of certain cell types (e.g., eosinophilic leukocytes).^{2, 48} sIgA in human BALF has been shown to be locally produced and is enhanced in bronchoalveolar fluid in patients with ABPA and appears to be specific for *A. fumigatus* antigens.⁴⁴

Phagocytosis and killing by phagocytic cells, such as alveolar macrophages and polymorphonuclear cells (PMN) in the epithelial lining fluid, constitute an important innate defense function of the airways. The phagocytic cells bear surface receptors that can recognize surface structures of microorganisms in a nonadaptive way: the so-called "pattern recognition receptors" (PRR).⁶⁰ An important surface structure of fungi is fungal mannan, which is recognized by the mannan-binding protein present on phagocytic cells.^{21, 105} The efficiency of phagocytosis and subsequent killing is improved by opsonization with sIgA and the various glycoproteins (described previously) that are present in the epithelial lining fluid. Recently, it has been shown that the surfactant proteins A and D are able to give help to the phagocytosis and killing of conidia of *A. fumigatus*.⁵⁵ After binding and phagocytosis, alveolar macrophages excrete proinflammatory cytokines, such as interleukin (IL)-1, IL-6, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α , that will prime and activate different cells in the environment (epithelial cells, polymorphonuclear cells). These cytokines are able to augment the phagocytic and killing capacity of phagocytic cells and induce an

increasing of the oxygen radical production.^{12, 20, 73} Furthermore, IL-1, TNF- α , and IFN- γ excreted by macrophages and dendritic monocytes are able to activate epithelial cells, inducing the production of chemokines, such as IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and RANTES, that will result in a second wave of cell recruitment (mononuclear and polymorphonuclear cells) in order to eliminate the microorganisms from the infected site (see Murali et al's article elsewhere in this issue).¹⁶

The importance of the innate immune defense has recently been supported by observations in a SCID mouse model, showing that even in the absence of functional T cells, an effective defense against fungi is found, provided that IL-12 had been administered.¹¹⁴

A. FUMIGATUS AND IMPAIRMENT OF THE MUCOSAL DEFENSE OF THE AIRWAYS

Different mechanisms account for the marked pathogenicity of *Aspergillus* species in the human host. Identification of the various virulence factors of *Aspergillus* over the past decades have revealed a bewildering array of mechanisms by which the fungus is able to interfere with or even block normal functions of the humoral and cellular defense of the airways. Some of these factors we discuss here briefly (Fig. 4).

1. Interference with the efficient clearance of impacted material in the airways, by decreasing the ciliary beating frequency, is the first level that can be modulated by substances excreted by microorganisms,⁵ including substances from *Aspergillus*. It has been shown that substances secreted by *A. fumigatus* are able to induce slowing and disorganization of the cilia beating frequency (CBF). Impairment of the CBF by *Aspergillus* will result in enhancement of the retention time of the microorganisms in the airways.^{3, 4}
2. The impairment of the fungicidal proteins present in the alveolar lining fluid may present the second level of attack by microorganisms. Presently, not much is known about the capacity of *A. fumigatus* to interact with these proteins, although it has recently been shown at our laboratory that *Aspergillus* protease activity is able to deactivate antileukoprotease by splitting a 2 kD fragment from the antileukoprotease molecule, rendering it unable to function as a protease inhibitor (Hiemstra and Tomee, unpublished observations). As previously discussed, antileukoprotease has antifungal activity, at least in vitro^{98, 100} and (partial) digestion of antileukoprotease by fungal products in vivo or as a consequence of local inflammation in the airways, resulting in release of products such as neutrophil elastase¹⁰⁸ that may impair the antifungal capacity of the mucus layer.
3. Interaction with the groups of opsonizing molecules has only been studied for the complement system. *A. fumigatus* antigens have been shown to be able to inactivate the complement sys-

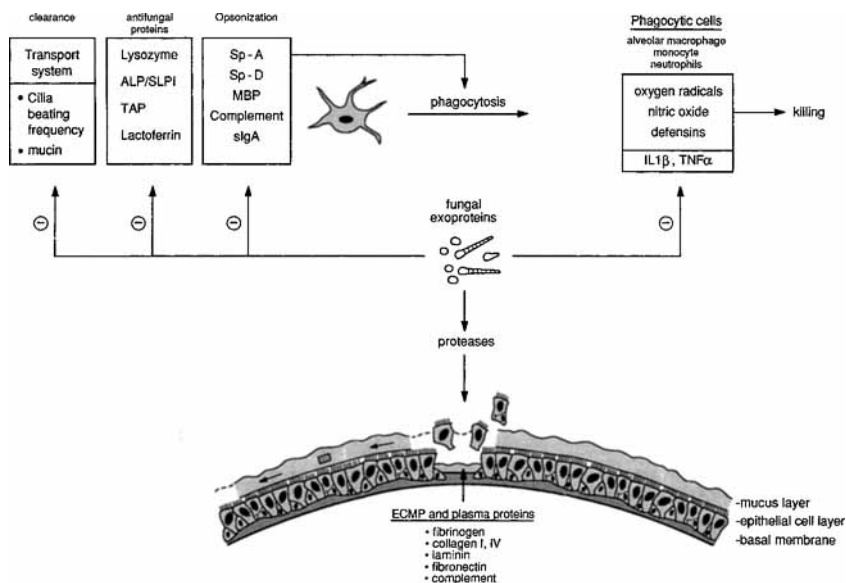


Figure 4. The pathogenicity of *A. fumigatus* and the innate airway defense. This figure summarizes the interference of *A. fumigatus* at the different innate defense strategies of the airways as described in Figure 2. In addition to a direct inhibition at the different defense systems, *Aspergillus* may also release proteases for a direct attack on the epithelial cells, causing detachment of epithelial cells. The damage of the epithelial cell layer will induce repair mechanisms with influx of plasma proteins and production of extracellular matrix proteins (ECMP) (lower part of the figure).

tem.⁹⁴ Furthermore, IgA antibody immune complexes appear to be of importance for the activation of eosinophilic leukocytes.^{2-18, 48} Interactions of the other components of *A. fumigatus* with different opsonizing molecules have not been studied and need further exploration.

4. An important observation is the capacity of *Aspergillus* to interfere with both the phagocytosis and killing capacity of phagocytic cells.⁸¹ Spores of *A. fumigatus* surviving ingestion by alveolar macrophages and have been described.⁵² Interference with this cellular defense system appears in part to be caused by soluble factors released by freshly isolated spores that are able to inhibit both the phagocytic properties as well as the capacity to suppress the oxygen radical production by phagocytic leukocytes.^{79, 80} The "conidial inhibitory factors" that are involved in this survival strategy are only, in part, characterized.⁶² In addition to factors that interfere with the phagocytosis and killing capacities of phagocytic cells, *A. fumigatus* also interferes with the proinflammatory signaling by alveolar phagocytes, such as IL-1 and TNF. These cytokines are released by these phagocytic cells and can

activate other cell types, such as epithelial cells and infiltrating PMN cells to participate in the cellular defense. Factors released by fungal spores or mycelium, such as fungal gliotoxin, are able to inhibit the production of these proinflammatory cytokines by inhibition of the nuclear transcription factor NF κ B via binding to the I κ B factor.⁶⁹ Also, the defensive activities of polymorphonuclear cells are inhibited in a similar way.⁶⁵ More recently, it has been shown that the low molecular weight component from the spores of *A. fumigatus*, responsible for the inhibition of the macrophage oxidative burst, is distinct from gliotoxin and other hyphal toxins.⁶²

5. During the last few years it has been found that certain strains of *A. fumigatus* are able to release proteolytic enzymes with elastolytic and collagenolytic activities.^{55, 78} The possible role of these proteolytic enzymes as a pathogenic factor in more invasive forms of aspergillosis is still a matter of debate.^{50, 51, 106} Findings in patients with ABPA or aspergilloma, however, indicate that these proteases may be involved in the pathogenesis of these diseases.^{64, 76, 99, 101} More recently, some new member of this family of proteolytic enzymes released by *A. fumigatus* was identified.^{10, 11}

EVIDENCE FOR THE ROLE OF PROTEASES IN ABPA AND ASPERGILLOMA

Several studies have reported on the antigenicity of protease from *Aspergillus* in the human immune response.^{34, 40, 63, 77} Results from our laboratory have indicated that proteolytic enzymes from *A. fumigatus* may actually be involved in the pathogenicity of ABPA and aspergilloma. In these studies, we obtained culture filtrate extracts with marked elastase and collagenase activity by culturing a human isolate of *A. fumigatus* on a collagen-based medium.⁹⁹ Using these collagen culture fluid extracts in Western blotting experiments using pooled sera from patients with ABPA or aspergilloma, binding of IgG antibodies to a 32 kD protein was found. Fractionation of the culture filtrate extracts by gel filtration and ion-exchange techniques revealed a high association between the fractions with high elastase activity and the 32 kD protein, indicating the protease nature of the 32 kD protein in our experiments. The pronounced binding of IgG antibodies with the 32 kD fungal elastase suggest that these proteases are produced *in vivo* in patients with ABPA and aspergilloma. Furthermore, it was found that during exacerbations of aspergilloma, antibody concentrations against different antigens including the 32 kD and 40 kD proteases were markedly increased, whereas IgG binding to the 32 kD protease, although somewhat suppressed, was also observable during remissions of the disease.¹⁰¹ Following antifungal treatment with itraconazole, the patient improved clinically, coinciding with a marked decrease in antibody concentration against the 32 kD and 40 kD proteases, reaching pre-exacerbation levels.

The observation that the antibody concentrations against the 32 and 40 kD fungal proteases is increased during the exacerbation phase indicates that these proteases may play a role in the pathogenicity of the disease. Evidence for the role of the newly described proteases must await further studies.

A. FUMIGATUS AND INTERACTION WITH THE EPITHELIAL CELLS: IMPLICATIONS FOR ALLERGIC ASTHMA AND ABPA

Role in the Induction of the Inflammatory Response

An important feature of pathogenic microorganisms is their capacity to interact with epithelial cells of the mucosal surface. Previously it has been shown that products released *in vitro* by *A. fumigatus* are able to cause epithelial cell detachment.^{82, 107} In addition to direct attack by proteolytic enzymes, spores of *A. fumigatus* are attached to epithelial surface cultured *in vitro*.⁷⁰ Whether this binding of spores to epithelial cells is an additional part of the defense system of the airways or reflects a first step to infection is as yet unclear. One study showed that attachment of spores to the epithelial surface is followed by phagocytosis by the epithelial cell, suggesting a defensive role,⁷⁰ whereas a second study showed that binding of spores to the epithelial surface is followed by germination and penetration of hyphae into the epithelial cell.¹⁷ It is interesting to note that the physical presence of *A. fumigatus* on and between the epithelial cells is possibly of importance for the modulation of the immunologic response towards a Th2-type response. It has been shown in mice models that particulate antigens of *A. fumigatus* will direct the response to more elevated levels of IgE and increased numbers of eosinophils both in blood and lung tissue than in animals exposed to soluble antigens.⁵³ Destruction of the epithelial cell barrier is rapidly followed by repair mechanisms, resulting in the influx of serum proteins and extracellular matrix proteins to the lumen site of the epithelium.⁷⁵ It has been shown that spores and mycelium of *A. fumigatus* have surface structures that are able to interact with extracellular matrix molecules (ECM) and serum factors such as fibrinogen, collagen I and IV, laminin, and complement C3.^{7, 13, 14, 28, 94, 95, 104} By rapid binding to the damaged epithelium, *Aspergillus* is able to bypass the sacrificial release of columnar epithelial cells, which is the first defensive strategy of the epithelial cell layer against infectious agents.²² Therefore, damage and concurrent repair mechanisms of the airway mucosa with concurrent release of these repair molecules may facilitate the binding of *Aspergillus* to the damaged sites of the airways. In this way, the elimination of fungus by cilium-mediated transport of the mucus layer is impaired.

In studies from our laboratory, we found that human bronchial and alveolar epithelial cell lines produced proinflammatory cytokines, such as IL-8, IL-6 and monocyte chemotactic protein (MCP)-1, after incubation

with protease-containing culture filtrates of *A. fumigatus*. This cytokine-releasing activity could be ascribed to the proteolytic activities of these extracts.¹⁰² These observations suggest that proteolytic enzymes released by *Aspergillus* growing on and between epithelial cells may be responsible for the induction of chemoattractive cytokines by epithelial cells.

Role of Eosinophils in ABPA

During exacerbations of ABPA a massive infiltration of eosinophils as has been described in BAL and biopsies from these patients.^{44, 91} It may be argued that this infiltration of eosinophilic leukocytes in the bronchial tissue of patients with ABPA will at least be caused in part by the *Aspergillus* protease(s)-induced release of chemotactic factors by epithelial cells.¹⁰² The function of this infiltration of eosinophils is uncertain. Although influx of invading phagocytes is generally functional as an additional help for the elimination of microorganisms, such a function of eosinophilic leukocytes in elimination of *Aspergillus* spores or mycelium has never been shown. Phagocytosis of suspended spores of *A. fumigatus* was found for both neutrophils and eosinophils with a similar capacity (Tomee, unpublished), but these activities have not been shown for eosinophils under in vivo conditions.⁹¹ Furthermore, it has been shown that infiltration of eosinophils in atopic asthmatic patients are the cause of additional damage to the epithelial cell layer by release of their toxic granular proteins, such as major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). Eosinophils in biopsies of patients with asthma were shown to be in a state of degranulation with deposition of their toxic granular proteins in the bronchial tissue (EG2 positive cells) after allergen-induced asthmatic responses.^{1, 74} This degranulation of eosinophils is associated with damage of the airway surface structures, such as detachment of epithelial cells.¹⁰⁹ As has been described previously, damage of the airway surface will promote the binding of *Aspergillus* to these damage sites. In this way, the infiltration and degranulation of eosinophils will induce an ongoing cycle of damage to the airway epithelial surface (Fig. 5) with additional binding and growth of *Aspergillus* on and between the epithelial cells. The additional and enhanced release of proteolytic enzymes and allergens on the epithelial surface will induce a continuous inflammatory response and mast cell degranulation resulting in severe and long-lasting periods of exacerbations of ABPA. The continuous release of antigens and allergens will induce a strong activation of the Th2-type immunologic response with very high productions of total and specific IgE antibody and an additional Th1 response with formation of IgG and IgA antibodies to antigens of *A. fumigatus*, as is observed in patients with ABPA (see Fig. 1). It is interesting to note that the model of direct epithelial cell activation followed by an eosinophilic inflammatory response as described previously (which is not directly dependent on IgE-mediated mast cell degranulation) is

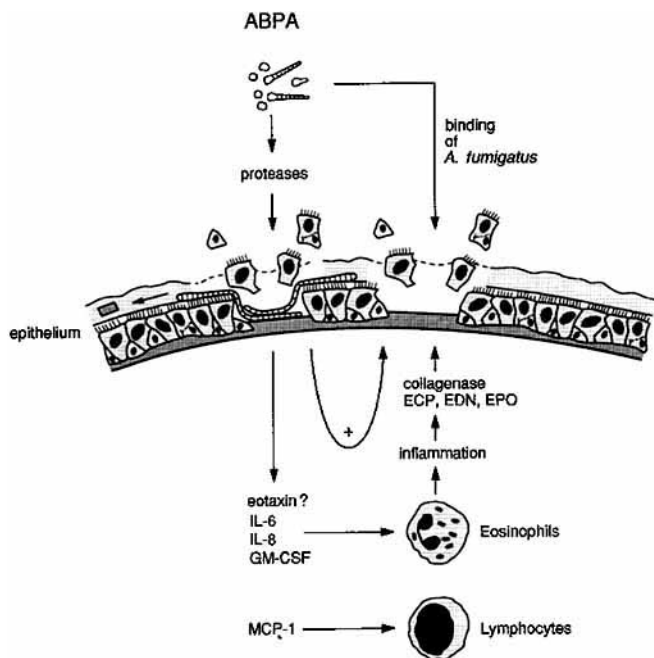


Figure 5. Inflammatory response caused by *A. fumigatus* in the airways of patients with ABPA. After the damaging effects of proteases, epithelial cells start to produce chemokines that attract eosinophilic cells and lymphocytes. The infiltration and degranulation of eosinophils causes increasing damage to the epithelial area, thereby opening additional binding sites for *A. fumigatus*, resulting in a positive feedback cycle of the inflammatory response. ECP = eosinophil cationic protein; EDN = eosinophil-derived neurotoxin; MBP = major basic protein.

consistent with recent observations in animal models, showing allergen-induced bronchial hyperreactivity and eosinophilic inflammation with *A. fumigatus* antigen in IgE-deficient mice that were previously sensitized with *Aspergillus* antigen.⁶¹

Although information is lacking on the further progress of damage of the airways of patients with ABPA, the continuous findings of bronchiectatic lesions, fibrosis, and hemoptysis during exacerbations of the disease suggest that the proteolytic components excreted by *A. fumigatus* together with the components from infiltrating eosinophils (collagenase, cytotoxic cationic proteins) may result in further damage of the airways. It is tempting to suggest that the cycling inflammatory responses as demonstrated in Figure 5 will result in strong damaging activities to the airway tissue in patients with untreated ABPA and may give an explanation for the fibrotic damages seen in patients with long-lasting ABPA (bronchiectatic lesions, sealing structures of the airway blood vessels, see Fig. 2).

GETTING CONTROL OF THE INFLAMMATORY RESPONSE: ROLE OF CORTICOSTEROIDS AND ANTI-INFLAMMATORY CYTOKINES

Although treatment of ABPA is described elsewhere in this issue (Fink's article), we discuss the implications of the actions of corticosteroids on the inflammatory model as described previously.

Having accepted the model of the self-destructive role of the eosinophilic inflammation in patients with ABPA favoring the growth of *A. fumigatus* in the airways, the mechanism of action of corticosteroids (CS) is more easily understood. CS have a strong capacity to inhibit the inflammatory responses in the airways. After treatment with prednisolone, a rapid decline of eosinophils has been observed both in the peripheral blood and in the bronchoalveolar fluid.^{31, 42, 43} The mechanism by which this rapid resolution of the eosinophilic inflammation is reached is still largely unknown. CS may act on the generation of eosinophils from the bone marrow, inhibition of the priming and activation of eosinophils in the blood circulation, prevent adhesion, and diapedesis into the airway tissue. More recently, it has been shown that, in contrast to the action on polymorphonuclear cells (neutrophils), CS induce an increase in apoptosis of eosinophils.⁵⁹ This latter mechanism may be responsible for the rapid resolution from eosinophils from the airway tissue as is found in patients treated for exacerbations of ABPA. By interruption of the damaging cycles of release of both proteolytic enzymes from *A. fumigatus* and from the eosinophils, the residual defense of the airway to fungi, appears to be sufficient for the elimination of *Aspergillus*, resulting in decreasing antibody titers (IgG and IgA), even reaching values comparable with healthy controls.⁴³ In contrast to IgG levels, the IgE titers, although declining to lower values, generally remain positive, possibly because of the daily low-level exposure to *Aspergillus* spores and antigens. It has been shown that Th2-type immune responses are activated at allergen levels considerably lower than concentrations needed for Th1-type immune activation.¹⁰⁶

Studies, mainly from animal models, suggest that antiinflammatory cytokines that promote the Th1-type defense system are able to suppress the damaging inflammatory responses as described previously (see Grünig et al's article). It was demonstrated that switching to immune response to a Th1-type expression by either administration of IL-12 (SCID mouse model) or blocking IL-4 with IL-4 antagonist together with an antifungal treatment (immunocompetent mouse model), resulted in an effective protective defense against fungi. The suppressive effects of IL-10, as demonstrated in a murine model,³³ and the suppressive effects of IL-10 on the oxidative burst of human mononuclear cells against *A. fumigatus* hyphae,⁸³ provide new possibilities for future treatments.

CONCLUSIONS

The innate immune defense of the airways is highly efficient in the elimination of fungi. The levels of mucosal strategies for elimination of

fungi were reviewed, and the different mechanisms that are used by *A. fumigatus* to impair these mucosal defense systems were described. It is argued that proteases released by *A. fumigatus* will cause damage to the epithelial surface, thereby inducing the release of cytokines, including chemokines. In atopic patients, these chemokines are responsible for the invasion of eosinophilic leukocytes. Activation and degranulation of eosinophils in the neighborhood of epithelial cells will result in additional damage to the epithelial surface. The concurrent repair reaction, with production of extracellular matrix proteins and serum factors, creates additional binding sites for *A. fumigatus*. In this way, a continuous inflammatory reaction with long-lasting growth of the fungus, extreme antibody formation, and additional infiltration and activation of eosinophils will be the cause of severe damage to the airways. The means by which corticosteroids and antiinflammatory cytokines are able to get control on this inflammatory response were described.

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