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Review

Catalases of *Aspergillus fumigatus* and Inflammation in Aspergillosis

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*This paper is dedicated to S. Paris who has recently retired from the *Aspergillus* Unit

Abstract

The article describs various features of aspergillosis and a discussed the role of calatases produced by *Aspergillus fumigatus* during infection. Since a large body of invasive *Aspergillus* infection occurs as an opportunistic infection in variously impaired defense mechanisms, there is a wide spectrum of histopathological features of lesions demonstrated at the site of infection. Accordingly, histopathology of the lesions can be understood as a phenotypical representation of interaction between differently impaired functions of neutrophils and macrophages and virulence factors of invading *Aspergilli*. Consideration of previous pathological knowledge regarding infection and inflammation provides much important information to predict the pathophysiology of a patient. Meanwhile, detoxification of hydrogen peroxide by catalases has been proposed as a way to overcome this host response. *A. fumigatus* produces three active catalases, one from conidia and two from mycelia. CatAp, a spore specific monofunctional catalase, is resistant to heat and metal ions. In spite of their increased sensitivity to H_2O_2, killing of *catA* conidia by alveolar macrophages, virulence in animals was similar to wild type conidia. In contrast to mycelial Cat1p, and CatAp catalases, the mycelial Cat2p is a bifunctional catalase-peroxidase enzyme and is also sensitive to heat, metal ions and detergent. Surprisingly, the mycelium of the double *cat1 cat2* mutant with no catalase activity has only a slightly increased sensitivity to H_2O_2 and was as sensitive to the killing of polymorphonuclear neutrophils as the wild type strain. However, it showed a delayed infection in the rat model of aspergillosis compared to the wild type strain. Consequently, it should be emphasized that conidial catalase is not a virulence factor but that mycelial catalases transiently protect the fungus from the host defence reactions.

Key words: catalase, aspergillosis, neutrophils, histopathology

Inflammation and fungi

The tissue repair, namely inflammation has been the hallmark of pathology. Knowledge of the basic phenomenon, as well as the consequences, complications, and nuances of this process, constitutes the basis for understanding the pathobiology of opportunistic fungal infections. Meanwhile, an injury agent or a damaged cell and normal inflammatory, homeostatic, and immune responses are the essential ingredients needed for inflammation to occur. Individual response to injury may vary widely with the injurious agent, owing to the unique set of genetic, nutritional, physical, infectious, chemical, hormonal, and immune factors that make up that individual's internal and external milieu. Inflammatory and reparative processes are generally simultaneous, but one phase usually dominates when tissue is examined microscopically at a given time after injury. Accordingly, invasive aspergillosis displays a specific inflammation caused by *Aspergillus* sp. as an injury agent[1)]. At the site of infection, alveolar macrophages and polymorphonuclear cells, cellular components of the innate defense

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of the lung, cooperate to control and eliminate the fungus in the airways. Macrophages eliminate conidia, and protection against the hyphal form is mediated by polymorphonuclear cells[2]. Reactive oxygen species produced by alveolar macrophages play an essential role in the killing of *A. fumigatus* conidia. Moreover, *in vitro* studies of neutrophil function have shown that hydrogen peroxide effectively kills fungal hyphae[3] and that neutrophil-mediated damage is blocked by the addition of a commercial catalase[4]. Therefore, features of inflammatory response including attacking cells and altered structure demonstrated at the site of infection must be generally epitomized by extensively complicated interaction between causative fungi and tissue response. In an opportunistic infection, especially in those with invasive aspergillus infections, tissue response against pathogenic fungi has been impaired, previously, but the cause and degree of decreasing function of defense mechanisms varied with the patient. Given that meaning, the feature of a lesion produced by an invasion of *Aspergillus* can be understood as a phenotypical expression emerging from an interaction between the invasiveness of the causative fungus and variously impaired defense mechanism of the host that is ubiquitously observed by microscope[1, 5]. In this article, histopathological spectrum of invasive aspergillus infection is described for better understanding of the relationship between the defense mechanism of the host and invasiveness of *Aspergilli*, followed by discussion on the role of catalases produced by *Aspergillus fumigatus*.

Variety of microscopic features in aspergillosis

Immunocompromised hosts have increased in number in recent years due to the increasing number of patients undergoing chemotherapy, HIV infection, organ transplantation, and long-term administration of an immunosuppressant. Under these circumstances, invasive fungal infections have been attracting public attention as opportunistic infections in the immuno-compromised hosts for many years[6–8]. The overall incidence of invasive fungal infections, especially *Candida albicans* at autopsies is now tending to decrease largely owing to the introduction of new triazole antifungal agents. In contrast, the proportion of aspergillosis in invasive fungal infections continues to increase. Invasive pulmonary aspergillosis is often diagnosed incidentally at autopsy because of the difficulty in diagnosing its earlier rapid progression, and restricted options of useful antifungals[9]. Furthermore, there are various background factors related to the difficulty of identifying this disease such as a paucity of clinical symptoms and chest x-ray findings, a low rate of isolation of fungi from sputum and other specimens obtained from the respiratory tract[10]. In addition, the general state of a patient is often so poor that invasive laboratory tests are restricted, making it even more difficult to establish a histopathological and cytological diagnosis.

It has been generally accepted that pulmonary aspergillosis of three types: allergic bronchopulmonary aspergillosis, fungus ball type (non-invasive) pulmonary aspergillosis, and invasive pulmonary aspergillosis[11]. Other clinical entities have been proposed, for example, chronic necrotizing pulmonary aspergillosis and semi-invasive form of aspergillosis; those are understood as a transitional form between the latter two types; non-invasive and invasive[12, 13]. However, the pathophysiological independence of the entity regarded as a transitional form has not been confirmed by histopathologically. A case initially diagnosed as pulmonary aspergillosis of fungus ball type characterized by non-invasive proliferation of hyphae in a preexisting cavity in the lung may transform into an invasive pulmonary disease when the defense mechanisms of the host are impaired by the innate course of the underlying disease and/or a requirement of induced immunosuppression as a therapeutic procedure. The non-invasive form of pulmonary aspergillosis has been termed aspergilloma and pathologically defined as development of a fungal ball in preexisting cavities usually caused by scar of earlier tuberculosis or cystic diseases of the lung. Hyphae compactly align in a radial pattern in the ball developed in the cavity the wall of which is usually covered with metaplastic epithelium of the respiratory tract or eroded. The inflammatory infiltrate observed in the wall essentially consisted with lymphocyte and plasma cells, and no hyphal invasion occurs when the patient is immunocompetent. From that meaning, the entity may be somewhat controversial because histopathological definition of the disease requires the exclusion of invasive fungal proliferation into the lung which is a necessory feature of the infectious disease. Understanding of the semi-invasive form of aspergillosis[12] is still confusing. However, this form is generally accepted as an intermediate stage between the non-invasive form into the invasive pulmonary disease when the defense mechanisms of the host are impaired by the innate course of the

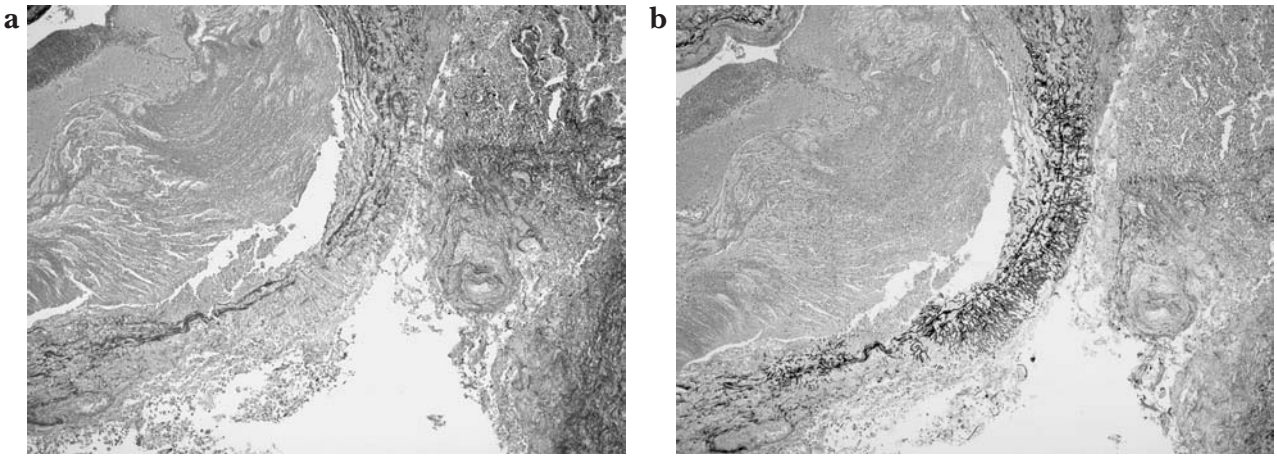

Fig. 1. Chronic necrotizing pulmonary aspergillosis.

a: Cavity wall is eroded with necrosis (Elastica stain, x100).
b: A pulmonary artery is involved by the invasion of *Aspergilli* (Elastica stain, x100).

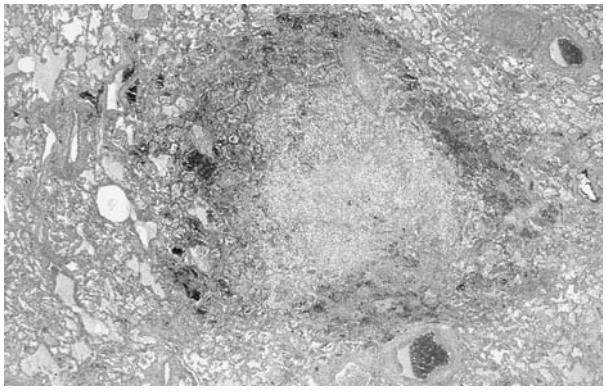

Fig. 2. Invasive pulmonary aspergillosis, discrete nodule.

There are sharply demarcated nodules comprising coagulation necrosis of the lung tissue surrounded by hemorrhage on a section of the lung (Hematoxylin-Eosin stain, x40).

underlying disease and/or a requirement of induced immunosuppression. The cavity wall is usually eroded and invaded by elongated hyphae. Both acute and chronic inflammatory infiltrates are seen association with fibrosis and necrosis in various degrees. Blood vessels are usually involved and occluded by the invasion may cause hemoptysis (Fig. 1).

Invasive pulmonary aspergillosis, the fatal form of the disease, can be classified in two patterns, which emerged from our previous study with 64 subjects examined at autopsy. One pattern consists in discrete nodule (DN) with of well-demarcated and round-shaped coagulation necrosis in which numerous hyphae aligned in a radial pattern (Fig. 2). A circumferential band of hemorrhage surrounds the area of coagulation necrosis. Less apparent in this pattern is inflammatory infiltrate that would usually occur in a patient with severe bone marrow suppression or agranulocytosis[10, 14]. A halo sign recognized as one of the important hallmarks of invasive pulmonary aspergillosis on CT image may mirror a band of hemorrhage surrounding DN developed in a patient with agranulocytosis. The second pattern was fused lobular consolidation (FLC), which corresponds to usual bronchopneumonia histologically characterized by filling of acute inflammatory exudates with a fungal proliferation in alveoli[5]. The gross feature of this pattern is a fusion of lobular consolidation (Fig. 3). Necrosis present in FLC is usually liquefation and may be induced by a neutrophilic infiltration (Fig. 4). This can produce a cavity at the center of the region when the bronchi involved by the necrosis have a role in the drainage. Patients indicated to have FLC retained a considerable response of neutrophils as their first line of defense against *Aspergillus* infection[14]. On the other hand, a cavity and peripheral air crescent may be caused by the exclusion of liquefaction necrosis produced by neutrophilic infiltrate against the invading fungi (Table 1). We have rarely encountered a patient who had provided us with insight into the pathogenesis of liquefaction. At the onset of pulmonary disease involving severe neutrophil dysfunction, a characteristic DN greater than 10 mm diameter could be identified on chest radiography. Later when chemotherapy was relaxed the white blood cell count recovered, and the patient died. In the postmortem examination, a macronodule of coagulation necrosis was identified. At its periphery, there was a zone of liquefaction necrosis containing a massive neutrophil infiltration and a tissue void with a spare margin of necrosis. The absence of necrotic tissue was probably the result of

Fig. 3. Invasive pulmonary aspergillosis, solid nodular consolidation.

The lesion composed of a fusion of solidified lobules is demonstrated on a section of the lung. Necrotic cavity is usually present at the center.

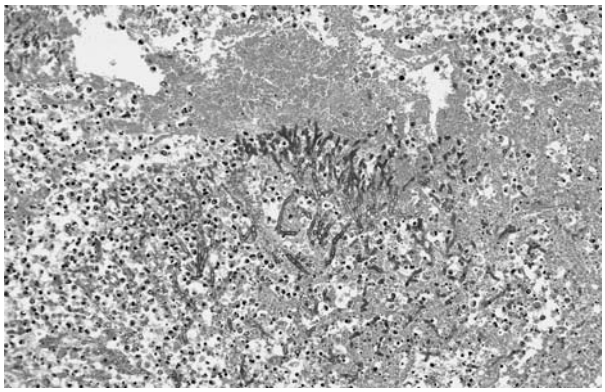

Fig. 4. Invasive pulmonary aspergillosis, solid nodular consolidation.

There is dense neutrophilic infiltrate with massive liquefaction necrosis surrounding invading hyphae (Hematoxylin-Eosin stain, x200).

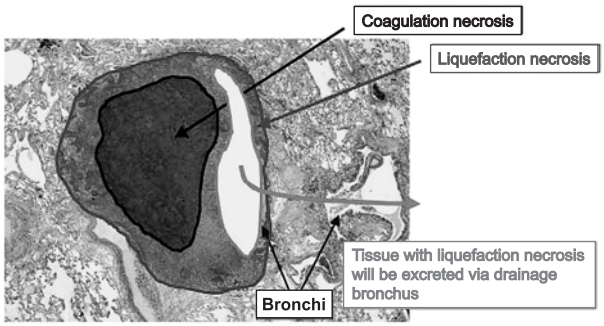

Fig. 5. Schematic representation of transition from DN to SLC in a patient with recovery of neutrophil function.

Crescent of cavity comprising sequestrum with neutrophilic infiltrate is shown at the periphery of nodule coagulation necrosis of the lung tissue (Original photograph: Hematoxylin-Eosin stain, x40).

drainage of liquefaction by nearby communicating bronchi (Fig. 5)

Role of catalases

Features of invasive aspergillosis were previously mentioned in this article in consideration of interaction between impaired defense mechanisms and invasiveness of *Aspergilli*. We here discuss another important subject comprising putative virulent factor *Aspergilli*. Alveolar macrophages and polymorphonuclear cells, cellular components of the innate defense of the lung, cooperate to control and eliminate the opportunistic fungal pathogen *A. fumigatus* fungus in the terminal airways. Macrophages eliminate conidia and protection against the hyphal form is mediated by polymorphonuclear cells[1]. Reactive oxygen species produced by alveolar macrophages play an essential role in the killing of *A. fumigatus* conidia. Moreover, *in vitro* studies of neutrophil function have shown that hydrogen peroxide effectively kills fungal hyphae[3] and that neutrophil-mediated damage is blocked by the addition of a commercial catalase[4]. For this reason, invasive aspergillosis does not occur in individuals who do not impaired defense mechanisms. Accordingly, catalase which is a good scavenger of H_2O_2, is considered to be a putative virulence factor of *A. fumigatus* that could counteract the oxidative defense reactions of the host phagocytes[15]. This article describes the role of the entire panel of conidial and mycelial catalases of *A. fumigatus* in the pathogenicity of the fungus.

There are three active catalases expressed by *A. fumigatus* which emerged from our study; one is present in the conidia and two in the mycelium that are encoded by three separate

Table 1. Variety of Pulmonary Infection caused by *Aspergilli*

Pathological Form	Invasiveness	Degree of Inflammatory infiltrate	Necrosis
Fungus Ball	−	Lymphocyte (+)	No necrosis
CNPA	+	Neutrophil (+)	Liquefaction (+)
IPA-SLC	++	Neutrophil (++)	Liquefaction (++)
IPA-DN	+++	None	Coagulation (++)

Abbreviations: CNPA: Chronic necrotizing aspergillosis, IPA-SLC: Solid lobular consolidation of invasive pulmonary aspergillosis, IPA-DN: Discrete nodule of invasive pulmonary aspergillosis

Table 2. Histopathological findings in rats infected by *Δcat1 Δcat2* mutant and G10 parental strain

	G10	*Δcat1 Δcat2*
(1) 5th day of infection		
Nodules	1 mm, confluent	0.5 mm, separated
Hyphal elongation	++	+
Neutrophil infiltrates	++	+
Nuclear debris	+	−
Macrophages	+	++
(2) 13th day of infection		
Necrosis	+++	+
Emboli	+++	−

Degree of each histological alteration is indicated from − to +++; −: not visible, +: present, but few or mild, ++: moderately demonstrated, +++: prominent or numerous

structural genes *CATA*, *CAT1*, and *CAT2*. CatAp is the only catalase present in resting conidia and is absent from hyphae. This unglycosylated catalase is very resistant to heat, denaturing agents and metal ions. CatAp was found to be a dimer[16, 17], whereas most large subunit monofunctional catalases are usually tetrameric[18]. The significance of this dimerization is unknown.

Our previous study elucidated that *ΔcatA* conidia were killed at lower doses of H_2O_2 than conidia of the G10 parental strain, but the killing by murine alveolar macrophages was identical for both G10 and *ΔcatA* conidia. So the conidial catalase CatAp, while protecting the spore against the deleterious effect of hydrogen peroxide *in vitro*, does not play any role in protecting conidia against the oxidative burst of macrophages that is known to play an essential role in the killing of conidia. This result suggest that the main ROS playing a role in the conidial killing by macrophages is not H_2O_2.

The mycelial catalase Cat2p has a peroxidase activity, a high electrophoretic mobility, is not glycosylated, and is very sensitive to heat in contrast to the Cat1p. So, Cat2p corresponds to the fast catalase-peroxidase described by Hearn *et al*[19]. Cat2p was found to be monomeric. This is surprising since most microbial catalase-peroxidases are active as either dimers or tetramers[18], and only two from halophilic bacteria were found to be monomeric[20]. The *CAT2* gene has no intron, a result that is atypical of the *A. fumigatus* ORFs sequenced so far. This absence of an intron was however also observed in the fungal catalase-peroxidase genes sequenced so far. Mycelia from single mutants were as sensitive to H_2O_2 as the wild type strain. These results are in agreement with similarity in virulence of the single mycelial catalase mutant and the wild type strain of *A. fumigatus* in immunosuppressed mice[21, 22]. The deletion of both *CAT1* and *CAT2* genes led to a slightly higher H_2O_2 sensitivity of mycelium and to a slower development of the mutant in the lungs of immunosuppressed rats. Thus, both catalases are needed to scavenge deleterious peroxide *in vitro* and in the rat model of infection (Table 2)[23]. However, the mycelial catalases are not sufficient to protect against the oxidative burst by immunocompetent human polymorphonuclear leukocytes (PMNL) *in vitro*. This suggests that mycelial catalases only provide a partial resistance to PMNL. One hypothesis to explain this residual resistance of *A. fumigatus* to H_2O_2 is the presence of additional catalases that may be specifically expressed during infection. Four other catalase genes (two *CAT1* and two *CAT2* homologs) have indeed been found in the *A. fumigatus* genome sequence TIGR database (http://www.tigr.org). However, this hypothesis is unlikely since neither catalase nor peroxidase activities could be detected in *in vitro* induction assays; when the $cat1^{-}cat2^{-}$ mutant was grown *in vitro* in the presence of subinhibitory concentrations of H_2O_2 (0.1-1 mM), no additional catalase was seen in our substrate gel assays. Another hypothesis is that H_2O_2 is not the primary ROS involved in hyphal killing and that other enzymes such as superoxide dismutase may be more efficient than catalases in protecting *A. fumigatus* mycelial growth against another ROS. Indeed, antigenic extracellular superoxide dismutases have been identified in *A. fumigatus*[24, 25], and could play an essential role in protection against ROS.

In conclusion, single and double mutants indicate that *A. fumigatus* conidial and mycelial catalases protect the fungus against hydrogen peroxide *in vitro*. However, while the conidial catalase CatAp is not a virulence factor, both

mycelial catalases, Cat1p and Cat2p, are involved in the degradation of hydrogen peroxide *in vitro* and transiently protect the fungus against the oxidative burst occurred in our experimental rat model[23]. Nevertheless, other oxidases are needed to overcome the host response. Since ROS have been shown to be essential for the killing of *A. fumigatus in vitro*, our *in vivo* results suggest that, in addition to H_2O_2, another ROS is needed for killing. The analysis of other oxidases required to overcome the oxidative burst *in vivo* may lead to the identification of those molecules that are essential for the killing of *A. fumigatus* by phagocytes.

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