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REVIEW ARTICLE

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Granuloma and cryptococcosis

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Abstract This review describes the general histopathological features of cryptococcosis in immunocompetent individuals, as well as in patients with acquired immunodeficiency syndrome (AIDS). Details of the histological examination of cryptococcal lesions are described, with the consideration of morphological modifications induced by treatment with highly active antiretroviral therapy (HAART). The essential histological features of cryptococcosis in individuals with impaired T-cell functioning are yeast-cell proliferation with a histiocytic response, but only minor lymphocytic and neutrophilic components. Several histological patterns of pulmonary cryptococcal lesions are introduced in this article, some of which could be graded with respect to the degree and type of inflammatory reaction. One pattern was a mild lesion consisting of scattered small foci of intraalveolar cryptococcal proliferation with a histiocytic response. Another pattern involved massive cryptococcal infection, which may have been simply more extensive than that in the mild lesion. Capillary involvement of alveolar septa should be understood as an important common finding in patients with AIDS who had not been treated with HAART. In those patients, the absence of T cells and a decreasing function of antigen-presenting activity in histiocytes were confirmed by immunohistological examination. These findings suggest that the lungs of AIDS patients without HAART offer little resistance to bloodstream dissemination by cryptococci. The unique histological feature demonstrated in patients treated with HAART is characterized by the presence of CD4+ cells, greater response of histiocytes and multinucleated giant-cell formation, and lack of massive capillary involvement.

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Inflammations, repair, and opportunistic fungal infection

Injury, inflammation, and repair are the hallmarks of pathology. Knowledge of the basic phenomena, as well as the consequences, complications, and nuances of these processes, constitutes the basis for understanding many diseases. Inflammation is the host-cell and tissue reaction to any injury. An injurious agent or a damaged cell, and normal inflammatory, homeostatic, and immune responses are the essential ingredients needed for inflammation to occur. Inflammatory responses may be provoked by many agents. The individual response to injury may vary widely for any given injurious agent, however, 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 any given time after injury. And the inflammatory response varies depending upon the particular agent, the tissue, and the individual host characteristics. Accordingly, an infectious disease can be recognized as an inflammatory response caused by a microorganism as an injurious agent.

The microscopic features of lesions demonstrated at the site of infection, and the altered structures, are generally epitomized by an extremely complicated interaction between the causative microbes and tissue response. In opportunistic infections, especially in those with invasive fungal infections, the tissue response to some pathogenic fungi may be impaired, but both the cause and degree of the decreasing function of defense mechanisms vary from case to case. Accordingly, the gross, microscopic, and ultrastructural features of the lesions produced by an invasion of pathogenic fungi can be understood as the phenotypical

expression that emerges from an interaction between the invasiveness of the causative fungi and variously impaired defense mechanisms of the host that are ubiquitously observed. We previously described the histopathology of invasive aspergillosis, with reference to variously impaired neutrophilic functions in hosts.[1,2] The inflammatory response to aspergillus can be essentially understood as an acute purulent inflammation that is characterized by necrosis and prominent neutrophilic infiltrate at the site of infection. Two types of necrosis, coagulation and liquefaction, are found in the lesions of invasive aspergillosis. When the disease occurs in patients with mildly impaired neutrophilic function, lobular consolidation develops at the focus of infection, and progressively enlarges. When the infection occurs in patients with severe neutrophilic dysfunction, the characteristic lesion is a discrete nodule with peripheral hemorrhage. If the patient has had continuous neutrophil dysfunction, the lesion consists of coagulation necrosis that can enlarge but does not change in morphology.[1] However, in previously neutropenic patients in whom neutrophil function has recovered, patent vessels around the discrete nodule will allow liquefaction necrosis to occur at the margins of the sequestered core of coagulation necrosis.[2] Cryptococcus, which is the main focus of discussion in the present review, is known as a fungus that causes a granulomatous response that is recognized as the phenotypical representation of the normal functioning of integrated cell immunity.[3] In these terms the defense mechanism against Cryptococci may be different from that against Aspergilli. In the present review, the histopathological features of the granulomatous response in several important forms of cryptococcal infection are described, and discussed, with reference to some particular mechanisms of the lowered functioning of immunity or defense mechanisms in the host, so that the pathogenesis as well as pathophysiology may be elucidated.

Architecture of granuloma induced by cryptococcal infection

In an immunocompetent individual, typical granuloma is usually encountered at the site of cryptococcal infection and is recognized as a compact aggregate of macrophages with epithelioid features and multinucleated giant cells, of both foreign-body and Langhans type, containing numerous intracytoplasmic yeasts (Figs. 1, 2, and 3). These cells are strongly positive for both HLA-DR and interleukin (IL)-1 beta. CD45RO-positive small round cells, corresponding to CD4+ lymphocytes, are visible in such typical granulomas.[4] When we observe experimentally induced cryptococcal lesions in athymic mice, the response of macrophages is confirmed, but they are not aggregated, and cryptococci are seen as both intra- and extracytoplasmic yeasts (Figs. 4 and 5). Although necrosis is rarely seen at the center of the granuloma, lesions with necrosis are essentially large. Thus, necrosis can be understood as a result of an ischemic state that is produced by an enlargement of the granuloma,

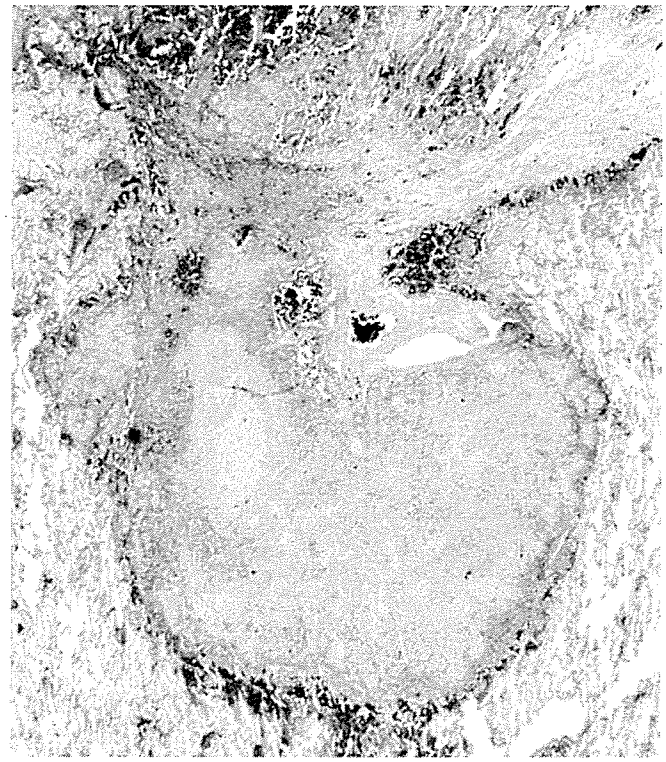

Fig. 1. Typical granuloma of cryptococcal infection in lung. H&E, ×4

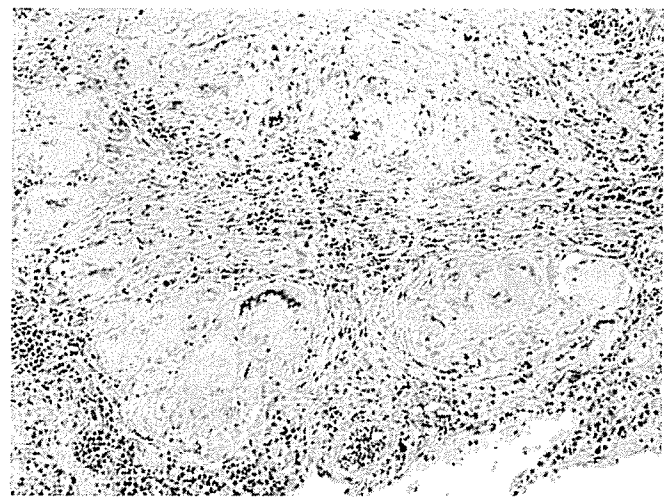

Fig. 2. Detail of the granuloma. The granuloma is composed of a compact aggregate of histiocytes with epithelioid features and multinucleated giant cells, of both foreign-body type and Langhans type. Periodic acid Schiff (PAS), ×200

and the proliferation of cryptococci itself does not causes necrosis.

Cryptococcosis and acquired immune deficiency syndrome (AIDS)

In the worldwide epidemic of AIDS, the pathological and clinical features, following infection by the human immuno-

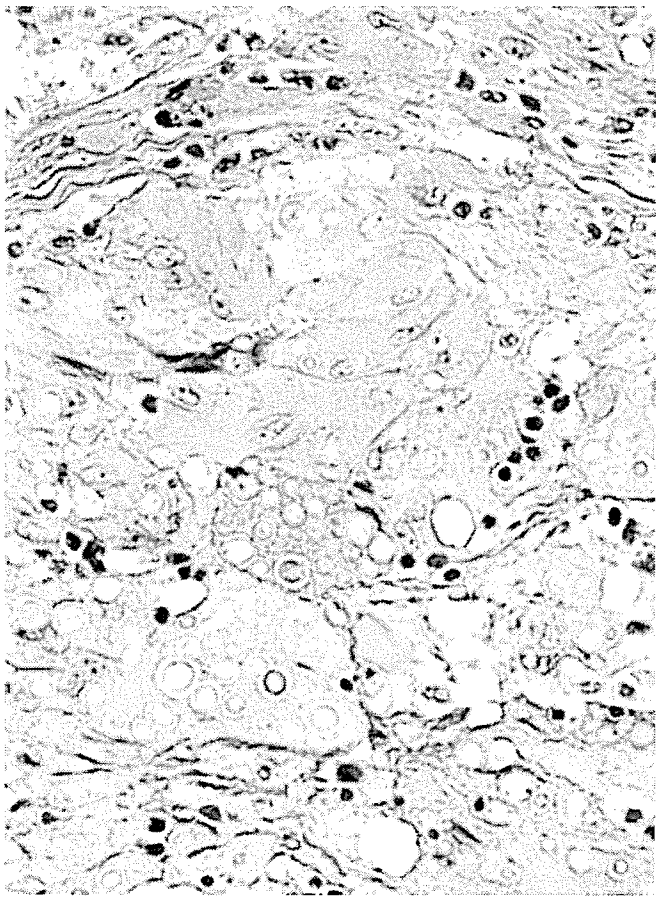

Fig. 3. Aggregate of multinucleated giant cells containing numerous phagocytozed yeasts. PAS, ×400

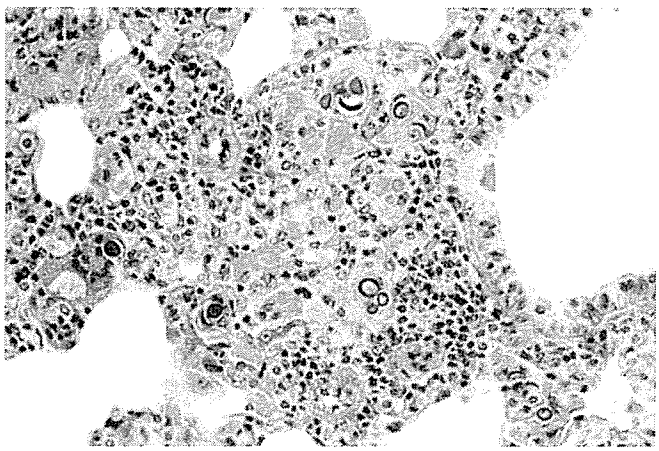

Fig. 4. Granulomatous lesion experimentally induced by an infection of cryptococci in nu/+ mouse. PAS, ×400

deficiency virus (HIV), are associated with a progressive decrease of cell-mediated immunity, due to defective functioning of CD4+ cells.[5,6] Because of this decrease in immunity, certain mycoses have risen dramatically in frequency, particularly systemic cryptococcosis. In AIDS patients, the incidence of opportunistic fungal infections, including any

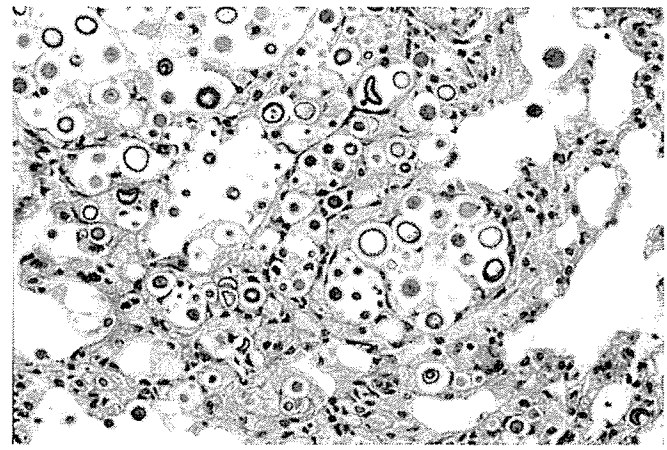

Fig. 5. Lack of granulomatous lesion in athymic mouse. PAS, ×400

species of fungus which provokes both localized and generalized disease, has been variously reported as 58%–81%, with 10%–20% of these patients having died as a direct consequence of the fungal infection.[7,8] It is well recognized that cryptococcal infection in AIDS patients often induces a fatal disease.[7,9,10] However, the acceptance of recent antiretroviral therapy in patients with AIDS has also had a dramatic impact on the epidemiology and clinical characteristics of many opportunistic infections associated with HIV.

Histopathology of cryptococcosis in patients with AIDS

Three patterns of pulmonary lesion have been reported.[11] The first pattern is small, consisting of intraalveolar proliferations of cryptococci with a histiocytic response. The architecture of the lung is unaltered, but involved alveoli are mildly expanded by both proliferating *cryptococci* and reacting histiocytes (Fig. 6). *Cryptococci* are prominent in capillaries (Fig. 7). However, the number of intraalveolar lesions varies from patient to patient. In the second pattern, foci are scarce, and there is focal proliferation of *cryptococci* with a major histiocytic response. *Cryptococci* are widely distributed in the lung, involving many alveoli, and are accompanied by histiocytes and multinucleated giant cells, which are loosely aggregated (Fig. 8). Most of the giant cells are of foreign-body type with fewer than ten nuclei per cell. Typical Langhans giant cells are not seen. *Cryptococci* are seen as extra- and intracellular yeast cells, with budding forms in both locations (Fig. 9). In the third pattern, there is a massive proliferation of cryptococci in both expanded alveoli and the capillary/interstitium, and septae are destroyed. A histiocyte and giant-cell response is present, as well as focal hemorrhage. Neither CD45RO-positive cells nor L26-positive cells are seen in the lesions of AIDS patients without highly active antiretroviral therapy (HAART), and the expression of HLA-DR and IL-1 beta is very weak, and rarely present in regenerated pneumocytes.

In patients who had been treated with HAART, consisting of zidovudine, lamivudine, and indinaver, however, a significant difference in histopathology, compared with findings in untreated patients, was recognized.[4] The pulmonary lesion was characterized by the presence of a lymphocytic infiltrate, a much greater response of histiocytes and multinucleated giant-cell formation, and the lack of massive capillary involvement. Although foci of dense cryptococcal proliferation were distributed throughout the lung, they were encompassed by fibroblasts and reacting histiocytes with considerable multinuclear giant-cell formation, but no giant cells of typical Langhans type were found (Fig. 10). In AIDS patients with HAART, CD45RO-positive cells are present at the periphery of each focus of dense cryptococcal proliferation. Both histiocytes and multinucleated giant cells are positive for HLA-DR as well as IL-1 beta, but their reactivity is relatively weak.

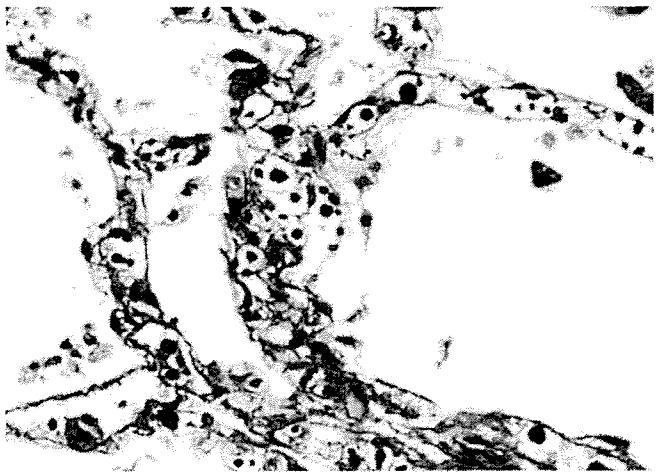

Fig. 6. Massive capillary involvement of cryptococci in a patient with AIDS without highly active antiretroviral therapy (HAART). PAS, ×400

Granulomatous response in AIDS patients with variously impaired T-cell function

Before the era of HAART, fatal opportunistic infections, such as cryptococcosis, mycobacterial infection, and pneumocystis pneumonia, usually developed in patients with AIDS. However, there were also very few patients with generalized candidiasis, and their pulmonary lesions showed prominent necrosis and neutrophilic infiltration. These facts support the idea that neutrophils play an important role in restricting candidal infection[6,16] in individuals with terminal HIV infection. Localized pulmonary aspergillosis also occurred in those with single-organ involvement, and also featured purulent bronchopneumonia with necrosis. Although it has been reported that the production

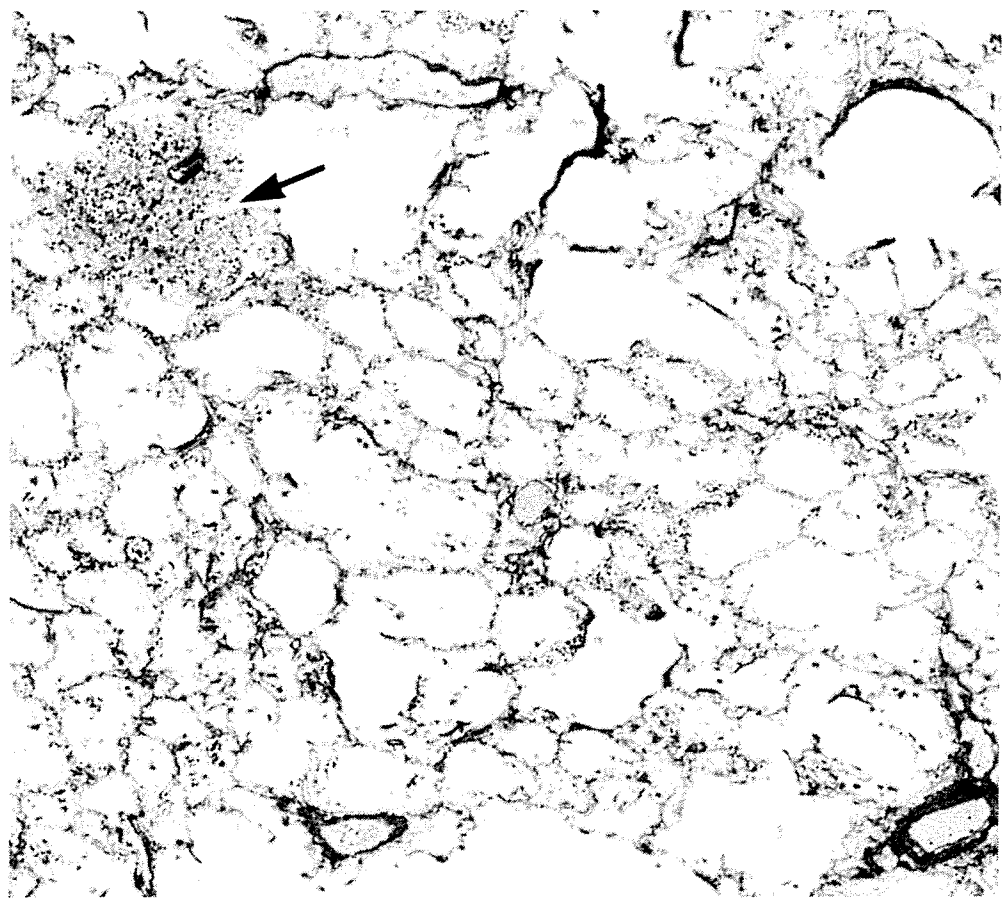

Fig. 7. A large part of the lung infected with cryptococci appears to have maintained air space, and there are a few minute intraalveolar foci (*arrow*) consisting of histiocytes. PAS, ×4

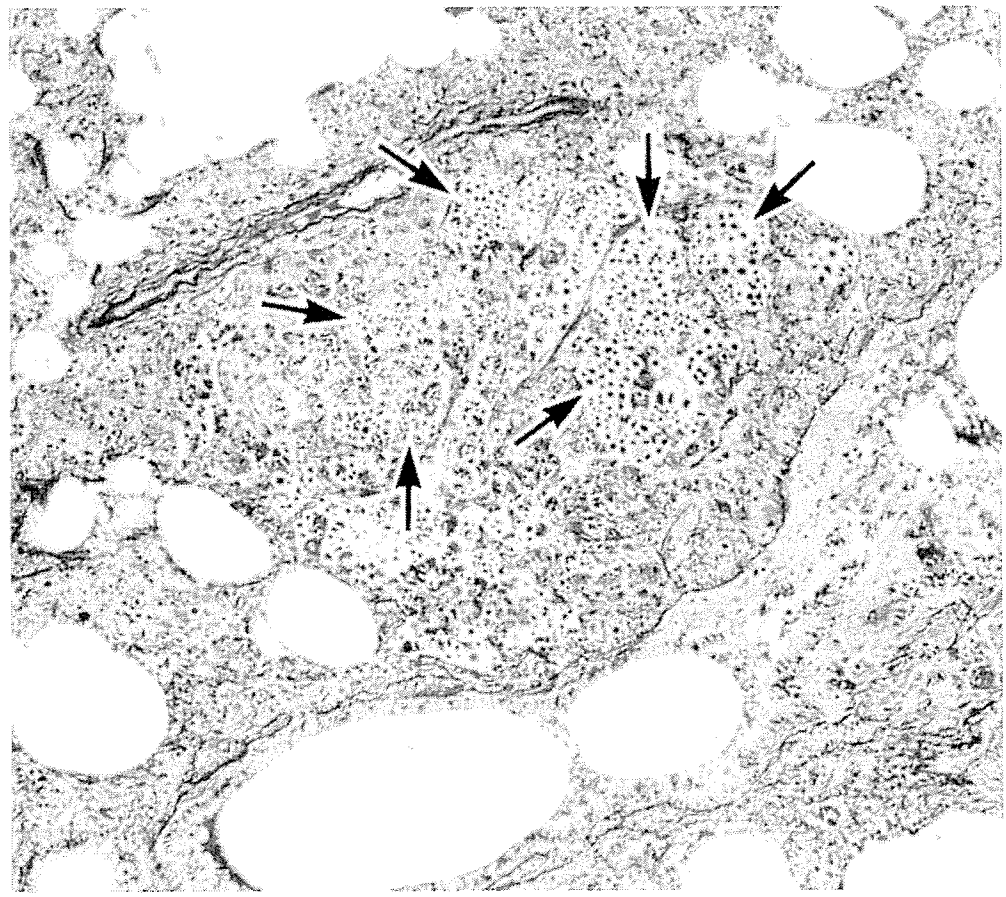

Fig. 8. Immature multinucleated giant cells of foreign-body type are present among histiocytes aggregated in alveoli; the cytoplasm in the histiocytes is markedly attenuated and enlarged by the intracytoplasmic proliferation of the yeast. (*arrows*). PAS, ×200

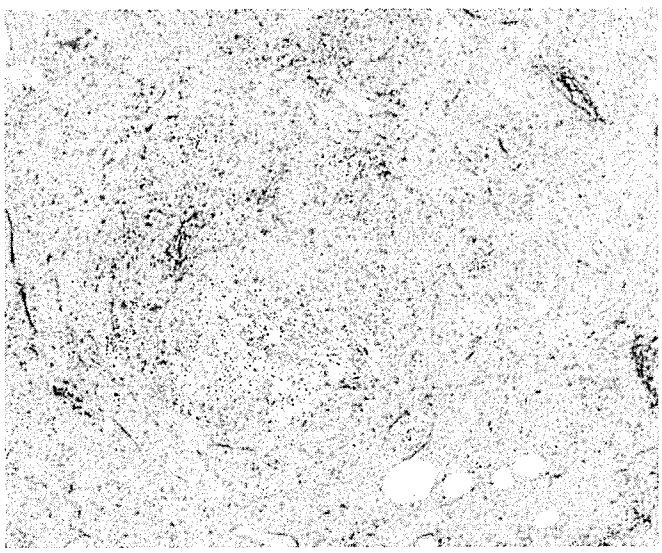

Fig. 9. Massive intraalveolar lesion of cryptococcal infection, consisting of proliferation *cryptococci* and reactive histiocytes (macrophages) without lymphocytic infiltrate. H&E, ×40

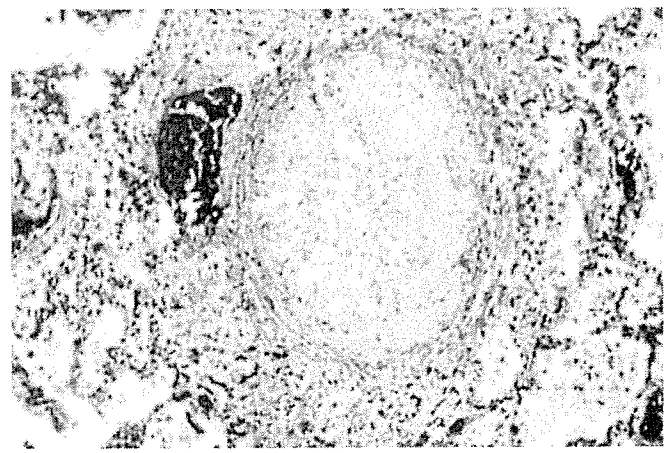

Fig. 10. Cryptococcal lesions that developed in a patient with AIDS treated with HAART. Scattered nodules are composed of dense proliferations of cryptococci, with the formation of marginal fibrosis, including histiocytes, multinucleated giant cells of foreign-body type, and an infiltrate of CD4+ cells. No capillary involvement is found in the septa, which are not involved by these nodular lesions. H&E, ×4

of IL-4 by CD4+ cells may be one major factor discriminating susceptibility and resistance to experimental *Aspergillus* infection,[16] few cases of generalized aspergillosis have been reported in AIDS patients. In patients with AIDS, the bloodstream dissemination of *Aspergillus* sp. appears to be prevented by the induction of a nonspecific purulent inflammation in the lung, the primary site of infection. This notion is supported by a previous report emphasizing that

the defense mechanism against *aspergilli* is mainly dependent on the functions of neutrophils and macrophages.[6,15] Thus, there is a striking histological difference between *Aspergillus* and candidial or cryptococcal infection in the lungs of patients with AIDS.

However, no purulent inflammatory responses were observed in the cryptococcal lesions of AIDS patients. This fact is supported by a previous investigation which concluded that cryptococcal polysaccharides, especially glucuronoxylomannan, can cause the shedding of L-selectin from the surfaces of neutrophils, and this may prevent neutrophils from attaching to the endothelial cell surface.[17] On the other hand, it has been reported recently that eosinophils may be one of the effector cells against *Cryptococcus neoformans*,[18] and tissue eosinophilia was experimentally induced in the lungs of mice infected with this organism.[19] In these animals, depletion of CD4+ cells ablated IL-5 production by lung leukocytes in vitro and eosinophil recruitment in vivo.[19] However, no cryptococcal lesions in humans have been associated with eosinophilic infiltration, and this is consistent with the depletion of CD4+ cells in HIV infection.

In immunocompetent hosts, cryptococcal infection has been recognized as a primary deep-seated fungal infection. The disease is usually asymptomatic,[20] and, although typical granulomas develop in the lung, this type of infection is thought to be self-limiting and benign.[12] However, the incidence of opportunistic cryptococcal infection has been rising in recent years, due in large part to increasing numbers of immunocompromised patients.[5,7,21,22] Before the HAART era, infection with *Cryptococcus neoformans* was a life-threatening disease often occurring in patients with AIDS.[10,21-25] In the past, many studies of cryptococcal infection in patients with AIDS have been reported, most of them concerned with clinical, microbiological, and immunological aspects,[5,8-10,23-28] but few were concerned with the histology of the human diease.[9,22,29] Four distinct histological types of pulmonary cryptococcosis have been classified by McDonnell and Hutchins;[12] peripheral pulmonary granuloma, granulomatous pneumonia, intracapillary/interstitial involvement, and massive pulmonary involvement, without special reference to a specific underlying disease.[2] Cryptococcal infection of the lungs in patients with AIDS took the form of intracapillary/interstitial or massive pulmonary involvement.[12,30] Peripheral granulomas and granulomatous pneumonia were not encountered in patients with AIDS, in whom the majority had lung lesions characterized by alveoli containing proliferating *cryptococci*, reactive histiocytes, and multinucleated giant cells, and organisms were not seen proliferating within the bronchial mucosa. On the other hand, capillary involvement was ubiquitously demonstrated in the lesions, whereas organisms were limited to relatively few alveoli. The lung is commonly considered as the portal of infection,[31,32] and might be expected to reflect this by manifesting an intraalveolar proliferation of inhaled yeasts without capillary involvement. However, we experienced five AIDS patients with generalized disease in whom the histological features were characterized by a few lesions showing the intraalveolar proliferation of *cryptococci* and widespread intracapillary involvement, without fibrous thickening of involved septae. In such patients, the intracapillary involvement may represent the hematogenous dissemination of inhaled yeasts, to which an extremely weak inflammatory response might be induced in alveoli in patients with terminal HIV infection. It has been reported that acute-phase mortality from cryptococcosis among AIDS patients with pneumonia was 42%.[10] Thus, the evidence of such a pattern is most likely explained by the rapidity of onset of vascular involvement, also leading to generalized disease. In addition, the histological alteration of such a pattern might be expected to manifest as a normal chest roentgenogram, and this has been reported as a common roentgenographic finding of pulmonary and/or generalized cryptococcosis in patients with AIDS.[28]

On the other hand, there is a striking difference in the histological features of cryptococcal lesions in AIDS patients with and without HAART. In those with HAART, these features can be summarized as: the presence of lymphocytic infiltrate, much greater response of histiocytes and multinucleated giant-cell formation, and lack of massive capillary involvement. This pattern may be transformed, from the massive capillary involvement that may have been previously produced in the patient by primary cryptococcal infection, as a sequel to the administration of HAART. In fact, in AIDS patients with HAART, Cd4+ cells were visible at the periphery of each nodule, consisting of dense cryptococcal proliferation. Accordingly, the recovery and reactivation of CD4+ cells induced by HAART can activate the histiocytic response to *cryptococci*, with prominent multinucleated giant-cell formation (Fig. 11). A hallmark of infection with *Cryptococcus neoformans* is depression of the immune system, characterized by poor inflammatory responses and loss of delayed-type hypersensitivity and antibody responses.[33] In all seven immunocompetent individuals that we examined, we found discrete granulomas, consisting of compactly aggregated giant cells and histiocytes that were strongly positive for HLA-DR as well as IL-1 beta, most likely as a sequel to the normal functioning of all considerable defense mechanisms against *cryptococci*. The development of a T-cell-mediated pulmonary inflammatory response is critical for the clearance of *cryptococci*,[34] and this has been demonstrated in murine cryptococcosis and is supported by data from several human studies.[6,34,35] Although it has been suggested that humoral immunity was elicited by a cryptococcal capsular polysaccharide,[36,37] none of the histopathological hallmarks of activated humoral immunity, e.g., reactive lymphadenitis and lymphoid follicular hyperplasia of the bronchial mucosae, have been shown in experimented studies.

The absence of CD4+ cells in pulmonary lesions has been confirmed, using immunohistochemistry, in patients with advanced HIV infection, and the recovery of these cells is the focus of HAART. Furthermore, the expression of IL-1 beta and HLA-DR was weak in histiocytes and multinucleated giant cells in immunocompromised individuals, when compared with granulomatous lesions in immunocompetent individuals. Alveolar macrophages are recognized as the first line of defense against cryptococcal

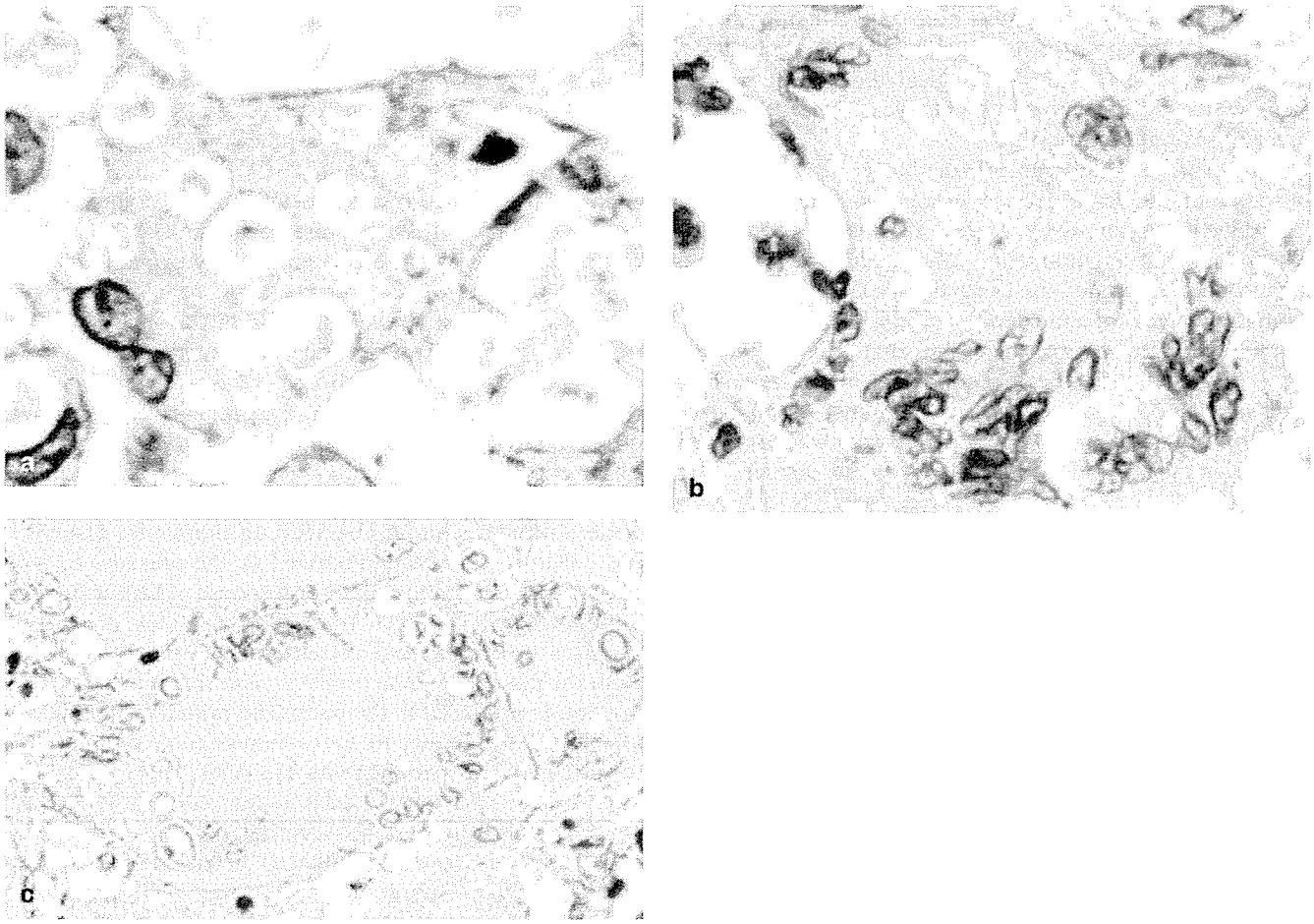

Fig. 11a–c. Comparison of features of multinucleated giant cells in patients with and without HAART, and a non-immunocompromised patient. **a** Giant cells in a patient with advanced HIV infection without HAART. There are many yeast cells in the cytoplasm of immature multinucleated giant cells of foreign-body type. **b** Giant cells in a patient with HIV infection with HAART. There is an increased number of nuclei in the cytoplasm of multinucleated giant cells of foreign-body type, which are more eosinophilic than those shown in **a**, and seem glassy. **c** Giant cells in a patient without any type of immunodeficiency. The multinucleated giant cell features are of the mature Langhans type, in which the nuclei are aligned in a single line at the periphery of the cytoplasm. **a**, **b**, **c** H&E, ×400

infection, and it has been reported, by Vecchiarelli et al.,[38] that human alveolar macrophages from normal subjects play a significant role in antigen presentation to T cells, while their effector function seems to be less relevant, at least in the afferent arm of the immune response to this yeast. That study was consistent with the decreased antigen-presenting activity of histiocytes in pulmonary cryptococcal infection (shown in their later study[39]), which in turn reduces the number of T cells in the lesion, consequently induced by HIV infection. The important event, however, from the findings of their later study,[39] is that the phagocytic activity of histiocytes reacting towards *cryptococci* was unaffected in AIDS patients, and phagocytosis was commonly present. This histological characteristic may be supported by a previous report indicating that bronchoalveolar lavage cells from early HIV-infected individuals did not have an intrinsic defect in fungistasis of *cryptococci*.[40] In addition to the lack of typical Langhans giant cells, the reactive histiocytes and multinucleated giant cells revealed that, while there was mostly normal phagocytic function, there was a decrease in the ability to kill *cryptococci*. The essential feature of the pulmonary lesions in patients with AIDS is the proliferation of *cryptococci* with reactive histiocytosis and a much lesser lymphocytic infiltration, which is, very possibly, the morphological response to cryptococcal infection in patients with manifest T-cell dysfunction. We wish to emphasize the absence of typical granuloma formation, the extensive capillary involvement, and the minimal lymphocytic response in cryptococcal disease in AIDS patients. Further, the reactivation of CD4+ cells induced by HAART can change the histological features of the cryptococcal lesions from predominant massive capillary involvement to granuloma-like formation in the presence of Cd4+ cells.

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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

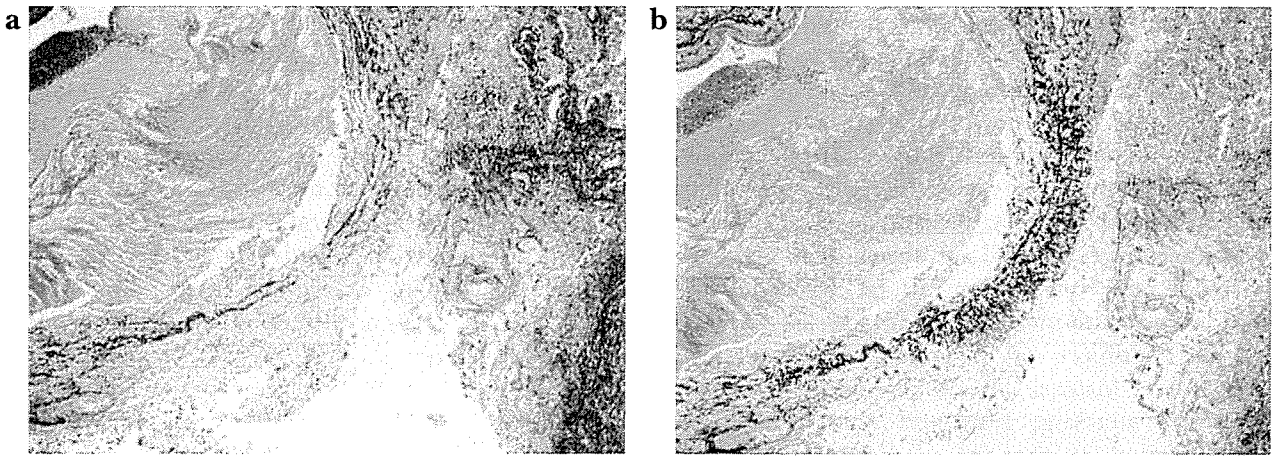

Fig. 1. Chronic necrotizing pulmonary aspergillosis.

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

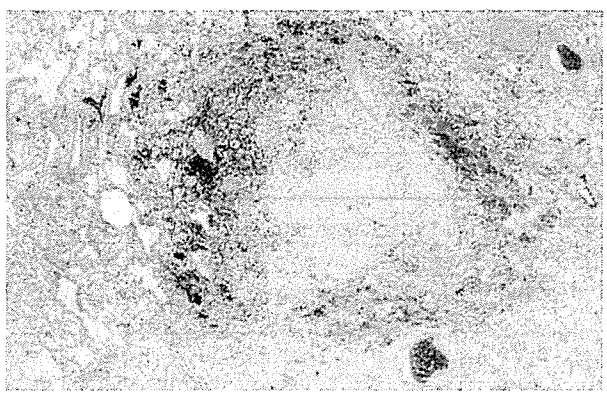

Fig. 2. Invasive pulmonary aspergillosis, discrete nodule.

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 broncho-pneumonia 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

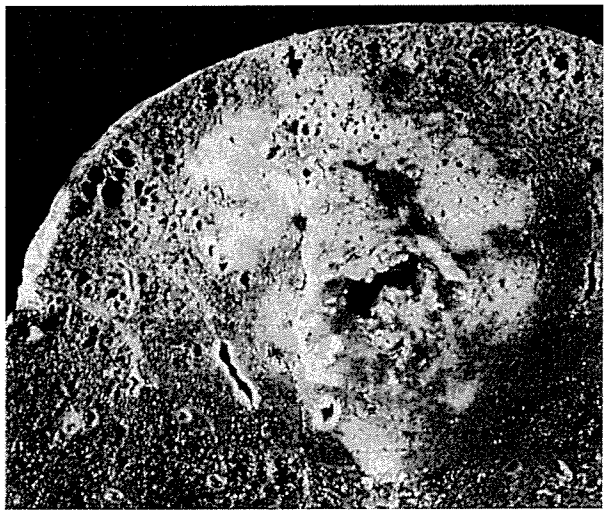

Fig. 3. Invasive pulmonary aspergillosis, solid nodular consolidation.

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.

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

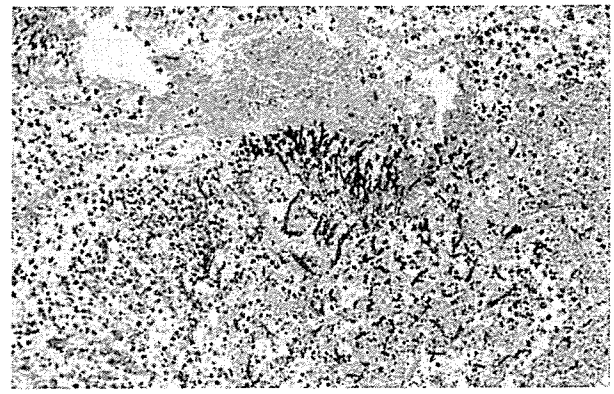

Fig. 4. Invasive pulmonary aspergillosis, solid nodular consolidation.

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

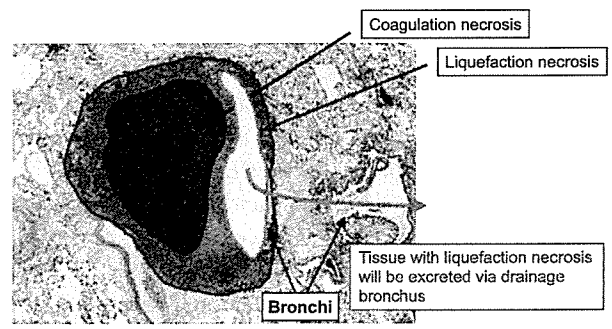

Fig. 5. Schematic representation of transition from DN to SLC in a patient with recovery of neutrophil function.

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).

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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Review article

Antifungal prophylaxis following reduced-intensity stem cell transplantation

Key words:
fluconazole; itraconazole; echinocandin; micafungin; voriconazole; hematologic malignancy; graft-versus-host disease; invasive fungal infection

Abstract: Reduced-intensity stem cell transplantation (RIST) has been developed to be a novel curative option for advanced hematologic diseases. Its minimal toxicity allows for transplantation in patients with advanced age or with organ dysfunction. Young patients without comorbidity can undergo RIST as outpatients. However, fungal infection remains an important complication in RIST. Given the poor prognosis of fungal infection, prophylaxis is critical in its management. The prophylactic strategy is recently changing with the development of RIST. Hospital equipment is important for fungal prophylaxis; however, the median day for the development of fungal infection is day 100, when most RIST patients are followed as outpatients. The focus of fungal management after RIST needs to shift from in-hospital equipment to oral antifungals. Various antifungals have recently been developed and introduced for clinical use. A major change in antifungal management will probably occur within several years.

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Allogeneic hematopoietic stem cell transplantation (allo-SCT) has been established as a curative treatment for hematologic malignancies. A myeloablative preparative regimen was considered essential to achieve sustained engraftment. Because of the considerable adverse events associated with the conditioning (1), allo-SCT has been restricted to young patients without organ dysfunction. In the 1990s, however, it was discovered that graft-versus-host disease (GVHD), a post-transplant immunological adverse event (2), is related to graft-versus-leukemia (GVL) effect (3). GVL effect is now considered to play a major role in tumor control after allo-SCT.

Progress in the study of GVL effect has revealed that myeloablative conditioning is not essential for engraftment

and tumor control (4). Consequently, a method of transplantation with a reduced-intensity conditioning has been developed. With the milder adverse events related to conditioning, the indication for reduced-intensity stem cell transplantation (RIST) has broadened to patients with advanced age or organ dysfunction. Young patients without comorbidity can undergo RIST in the outpatient setting. Not only hematologic malignancies but also a variety of diseases became possible indicators for treatment with RIST (5–7). Further studies evaluated RIST using unrelated donors (8, 9) and umbilical cord blood (10, 11), resulting in a steep increase in the number of candidates for RIST.

Fungal infection in the context of allo-SCT

Fungal infection is a major complication associated with allo-SCT (12). Although *Candida albicans* had long been the most common infectious organism, non-*albicans Candida* now accounts for more than half the cases of candidal infections (13). Cases of other fungi, such as *Aspergillus*, *Fusarium, Zygomycetes*, and *Trichosporon* are also increasing (14, 15). Notably, the number of cases of invasive aspergillosis (IA) has increased (16, 17). Despite improvement in diagnosis and treatment for IA (18, 19), the mortality remains high (16, 17). Long-term survival rate with amphotericin B (AMPH-B), a conventional standard medication, is below 30% (16). The clinical presentations of IA have also changed; the development of IA shows 2 peaks in the time course, during neutropenia early after transplantation and then several months after transplantation. Recent trends show an increase in late IA, which now accounts for the majority of cases of IA (17).

RIST is associated with a shorter neutropenic period and less regimen-related toxicity. Early studies on RIST suggested its safety for elderly patients or those with organ damages (20). However, recent studies revealed that both humoral and cellular immunity are severely impaired in RIST recipients (21), and that no significant differences were found in immune recovery between patients receiving RIST and those undergoing myeloablative transplantation (22). Invasive fungal infection (IFI) is a significant complication in RIST as well as in myeloablative transplantation (23, 24). Kojima et al. (24) compared the clinical features of IA following RIST with those following myeloablative allo-SCT. The incidence of IA was comparable: 4.3% in myeloablative allo-SCT and 7.9% in RIST recipients. The mortality rate of IA following RIST is high, as is that following conventional allo-SCT (76% and 86%, respectively) (24). While previous studies on IA following myeloablative allo-SCT showed that the onset of IA is bimodal (25), the development of IA following RIST has shifted to late onset (23, 24). The clinical features of late-onset IA have not been fully elucidated, and pathogenesis of late-onset IA might differ from that of early-onset IA. Kojima et al. (26) reported that the airways are frequently involved in late-onset IA, and that patients with bronchopneumonia-type IA have poorer prognosis than those with angioinvasive IA. It is unclear whether the difference can be attributed to fungal or patient factors. Further studies using animal models and more clinical experience are necessary to investigate the pathogenesis of late IA following RIST.

Antifungal prophylaxis in allo-SCT

Once developed, IFI has a poor prognosis. Thus, the focus of fungal management has been prophylaxis (27). The US Centers for Disease Control and Prevention (CDC) proposed guidelines for preventing fungal infections in allo-SCT in 2000 (28), which have been commonly used in many countries. However, the situation has changed with the progress in transplantation, and some of the guidelines might be obsolete (29), requiring updates suitable to the current situation.

The risk of IFI varies among patients; risk factors include advanced age, GVHD, cytomegalovirus infection, and corticosteroid use (17, 23). Based on the risk factors, physicians have attempted to predict IFI and focus prophylaxis on high-risk patients (27).

The hospital environment is fundamental to antifungal prophylaxis. The prophylaxis of endogenous pathogens such as *Candida* is focused on transmission by health care providers; their compliance with the standard precautions including hand-washing is important. When in-hospital transmission is documented, identification of the infectious route using a molecular technique is recommended (30). The focus in prophylaxis of *Aspergillus* infections is hospital equipment. Sources of infection include air, dust, construction sites, air-conditioning systems, plants, crops,

spices, and carpet (28); chances of exposure can be reduced by frequent room-cleaning, isolation from the outside, and negative pressure around construction sites within the hospital. The CDC guidelines include air-conditioning systems such as high-efficiency particulate air (HEPA) filtration (28). As HEPA filtration eliminates 99.97% of 0.3 μm or larger particles in the air, it can eliminate *Aspergillus* spores and reduce the rate of IA (31). Waterborne *Aspergillus* infections have recently drawn attention (32). Fungi are prone to colonize wet places such as shower faucets; the colonized shower spreads fungi in the air, increasing the risk of inhalation by patients.

While infection management is obviously important in antifungal prophylaxis, the median day of IFI development subsequent to RIST is day 100 after transplant, and IFI most commonly develops during the period of outpatient treatment. IFI management in RIST therefore places more emphasis on the prophylactic administration of antifungal agents, rather than on improvement in in-hospital equipment and precautions. In recent years, various antifungal agents have been developed and applied to clinical use. Never before have such a large number of agents been developed at the same time in the field of fungal infection management, forecasting potential major changes in the coming several years (33, 34).

Overview of antifungal agents: emergence of novel antifungal agents

A total of 6 agents in 3 classes are widely used for fungal prophylaxis and treatment following allo-SCT: AMPH-B (polyene); fluconazole, itraconazole, and voriconazole (azoles); and caspofungin and micafungin (echinocandins) (Table 1). Other new agents, such as posaconazole, ravuconazole, and anidulafungin, are under investigation.

Polyenes

AMPH-B is a polyene. Since its approval in 1958, AMPH-B has been commonly used as the standard medication for IFI. However, the agent is considered to have remained the standard not because of its significant efficacy, but because no alternative agents have been successfully developed.

Summary of antifungal agents

	Polyene	Azoles			Echinocandin	
	Amphotericin B	Fluconazole	Itraconazole	Voriconazole	Micafungin	
Antifungal activities						
Candida	Effective except for *C. lusitaniae*	Effective except for *C. glabrata* and *krusei*	Effective except for *C. glabrata* and *krusei*	Effective	Effective except for *C. parapsilosis* and *guilliermondii*	
Aspergillus	Effective	Ineffective	Effective	Effective	Effective	
Fusarium	Ineffective	Ineffective	Ineffective	Effective	Ineffective	
Zygomycetes	Effective	Ineffective	Ineffective	Effective[1]	Ineffective	
Kinetic properties	Renal excretion	Renal excretion	Metabolized in the liver	Metabolized in the liver	Metabolized in the liver	
Interactions	No significant interactions	Immunosuppressants	Antacids and immunosuppressants	Immunosuppressants	No significant interactions	
Adverse effects	Renal damage, fever, chills, and shivering	Digestive symptoms and elevation of liver enzymes	Digestive symptoms and elevation of liver enzymes	Vision impairment	Impaired hepatic function	

[1]Minimum inhibitory concentration generally higher.

Table 1

A drawback of AMPH-B is its narrow therapeutic range. Treatment with AMPH-B frequently causes adverse reactions such as fever, chills, and rigor, as well as occasional hypoxia and hypotension. The most concerning adverse effect is renal damage, sometimes requiring hemodialysis. Prolonged administration of normal saline is recommended to prevent renal damage (35). AMPH-B also causes hypokalemia, hypomagnesemia, anemia, and impaired hepatic function. Despite these adverse effects, it has long been the standard medication for IFI, as it has a potent and wide antifungal activity, except for *Fusarium*, *Trichosporon*, *Scedosporium,* and *C. lusitaniae* (35). While it is unusual to develop resistance, cell membrane composition has rarely been reported to alter and become resistant to AMPH-B (36).

Intravenous administration of AMPH-B is useful for IFI prevention in high-risk patients after allo-SCT. Although patients with a history of IA were not candidates for allo-SCT, with advance in antifungal prophylaxis the indication has broadened to include such patients (37). A locally isolated lesion is recommended to be surgically removed before allo-SCT (38).

The prophylactic dose of AMPH-B is usually low, approximately 0.1 mg/kg. Serum concentrations of low-lose AMPH-B were twice the minimum inhibitory concentration (MIC) of yeast isolates from patients' oropharyngeal areas and low-dose AMPH-B significantly reduced the numbers of yeast colonization (39). Nephrotoxicity is not common at such a low dose, although infusion-related reaction can occur. The efficacy of prophylactic use of AMPH-B has been suggested in multiple pilot studies (40, 41). In a randomized, placebo-controlled study, the incidence of IFI was significantly lower in the group receiving prophylactic low-dose AMPH-B, demonstrating its efficacy (42). Two meta-analyses including allo-SCT recipients and neutropenic patients have demonstrated significant reduction in odds ratio of IFI in patients given prophylactic low-dose AMPH-B (43, 44).

Although AMPH-B inhalation had been considered ideal prophylaxis as local administration enabled locally high concentration (45), a randomized, placebo-controlled study failed to prove the efficacy (46). The exact reasons for prophylactic failure of aerosol AMPH-B remain unknown, although insufficient delivery of aerosol AMPH-B to the lower respiratory tract (27) or poor compliance with AMPH-B inhalation might be possible causes.

Intravenous AMPH-B provides effective prophylaxis for IFI, yet prophylactic AMPH-B cannot completely control *Aspergillus* infection. A meta-analysis on prophylactic low-dose AMPH-B for neutropenic patients including allo-SCT recipients failed to show significant reduction in odds ratio of IA (44). Low-dose AMPH-B has become less common as prophylactic fluconazole is now more widely used.

Lipid-soluble formulations of AMPH-B have been developed to minimize nephrotoxicity, and now 3 formulations are available: liposomal AMPH-B, AMPH-B colloidal dispersion, and AMPH-B lipid complex (47–49). Lipid complexes of AMPH-B are designed to enhance transfer to the liver and spleen and to decrease transfer to the kidney, reducing renal damage. However, they also reduce transfer to the lungs, requiring 5 times the dose of AMPH-B deoxycholate to secure the same tissue concentration in the lungs. Different results have been reported on antifungal effects of liposomal AMPH-B and AMPH-B deoxycholate (50, 51). The lipid-soluble formulation, which is relatively more expensive than AMPH-B deoxycholate and fluconazole, has been applied to prophylactic administration in some double-blind placebo-controlled studies. However, those studies revealed that liposomal AMPH-B at a dose of 1–2 mg/kg/day had no significant effect in the prophylaxis of fungal infections (52–54).

Azoles

Fluconazole and itraconazole have both been used clinically, but they are significantly different in terms of the pharmacokinetics and clinical profiles (55). In recent years, itraconazole oral solution (ITCZ-OS) and voriconazole have been developed and evaluated in clinical studies. Because azoles have few adverse events, they are promising for prophylactic use; however, some researchers have concerns about their significant drug interactions and emergence of resistant strains.

Fluconazole

Fluconazole, the first synthetic triazole, is available in oral and intravenous forms and has been the first-line drug for antifungal prophylaxis in allo-SCT. In terms of pharmacokinetics, it is highly water soluble and indicates linear metabolism. It provides good oral absorption with an absorption rate of higher than 80%. Rate of protein binding is 12% and transfer to the central nervous system is 60%.