Host-Dependent Patterns of Tissue Injury in Invasive Pulmonary Aspergillosis

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Abstract

Invasive pulmonary aspergillosis (IPA) is an important cause of morbidity and mortality in neutropenic, nonneutropenic, and other immunocompromised patients. We therefore compared the patterns of infection and inflammation among 3 cohorts of immunocompromised patients with profound neutropenia, nonneutropenic immunosuppression, and hematopoietic stem cell transplantation. Lesions of IPA in neutropenic patients and hematopoietic stem cell transplant (HSCT) recipients were similar and consisted predominantly of angioinvasion and intra-alveolar hemorrhage. The frequency of these histologic findings in neutropenic patients and HSCT recipients differed significantly from those of nonneutropenic patients ($P < .05$). It is noteworthy that even if HSCT recipients have normal peripheral blood neutrophil counts, there may be no influx into sites of infection. In the nonneutropenic cohort, lesions of IPA consisted mainly of neutrophilic and monocytic infiltrates and inflammatory necrosis. Thus, the status of innate host defenses contributes significantly to the histologic patterns observed in IPA.

Invasive pulmonary aspergillosis (IPA) is an important cause of mortality in immunocompromised hosts, particularly those with cancer and hematopoietic stem cell transplantation (HSCT).[1-5] *Aspergillus* species are ubiquitous filamentous fungi, which sporulate abundantly and release conidia into the atmosphere. These conidia can be inhaled and may reach the pulmonary alveoli. *Aspergillus* species seldom cause disease in people with apparently normal innate host defense mechanisms. The first line of pulmonary host defense is that of the pulmonary alveolar macrophages, which eliminate conidia by phagocytosis and intracellular killing. Recruited neutrophils, which are the second line of defense, kill hyphae that escape from conidial killing by macrophages.[6] However, when these cells are quantitatively or qualitatively impaired, the host becomes susceptible to the development of IPA.[6,7] Therefore, patients with neutropenia or dysfunction of neutrophils and/or macrophages, such as patients with hematologic malignancies, HSCT recipients, and patients with inherited immunodeficiencies, have an increased risk of developing IPA.

Berenguer et al[8] observed that the status of innate host defenses in neutropenic and nonneutropenic immunocompromised animals with experimental IPA significantly affected the histologic pattern of pulmonary injury. Persistently neutropenic animals with IPA histologically demonstrated angioinvasion, intra-alveolar hemorrhage, and pulmonary infarction. By comparison, nonneutropenic immunocompromised animals revealed a pattern of inflammatory necrosis but no significant angioinvasion, hemorrhage, or infarction.

Little is known, however, about the impact of innate host defense on the histologic pattern of IPA in humans. We therefore studied the patterns of infection and inflammation among 3 cohorts of patients based on the presence or absence of profound neutropenia.

Materials and Methods

Case Definitions and Clinical Review

The files of the National Institutes of Health Clinical Pathology Department, Bethesda, MD, were searched for cases in which lung biopsies or autopsies were performed on patients with IPA in the 12-year period between January 1991 and December 2002. The diagnosis of IPA was based on microbiology reports and lung histopathology demonstrating fungal organisms with branching septate hyphal forms morphologically compatible with *Aspergillus* species. All cases with histopathologically documented infection were then further reviewed in this study. Data on demographics, primary diagnosis, transplantation type, laboratory examinations, and treatment were extracted from the medical records of these patients. Cases fulfilling these criteria were categorized into 3 cohorts: (1) profound neutropenia (absolute neutrophil count, <500/μL [$<500 \times 10^9$/L] for more than 1 week) within a month before the biopsy or autopsy, (2) no history of neutropenia, and (3) HSCT. HSCT recipients had 2 periods of risk: (1) neutropenia following the preparative regimen and (2) graft-vs-host disease (GVHD) following engraftment.

Two blinded reviewers (T.S. and T.J.W.) microscopically assessed the biopsy and autopsy slides for presence of angioinvasion, hemorrhagic infarctions, intra-alveolar hemorrhage, granulomas, coagulative necrosis, and inflammatory necrosis. By using prespecified criteria, they reached complete concordance in classification of these histopathologic features.

Histopathologic Definitions

Angioinvasion is the presence of hyphal structure within the wall or lumen of arteries or veins **Image 1A**. Hemorrhagic infarction is defined as the presence of RBCs within a region of infarcted lung tissue. Intra-alveolar hemorrhage is the presence of RBCs within 2 or more contiguous alveoli **Image 1B**. Granulomas are defined as organized aggregates of mononuclear cells, macrophages, lymphocytes, and neutrophils with or without caseous necrosis. Coagulative necrosis is the presence of nonviable tissue characterized by eosinophilia and loss of cell membranes but with preservation of pulmonary cellular architecture. Finally, inflammatory necrosis is the presence of mixed neutrophilic and monocytic inflammatory cells associated with necrosis of pulmonary parenchyma **Image 2C**.

Processing and Culture for Fungus

Direct microscopic examination and culture for fungi were performed on bronchoalveolar lavage (BAL) and lung tissue specimens. A 0.4-mL aliquot of BAL fluid was mixed with 0.1 mL of Shandon Collection Fluid and spun onto microscope slides in a Shandon Cytospin 4 Cytocentrifuge (Thermo Electron, Waltham, MA) for 10 minutes at 1,200*g*. Bloody or mucoid specimens were treated with Mucolyse (1:2 ratio; Pro-Lab Diagnostics, Richmond Hill, Canada) and heated at 35°C for approximately 3 minutes before cytocentrifugation. From tissue specimens, slides were made from touch preparations and homogenized samples. Following methanol fixation, slides were stained with Fungi-Fluor Staining Solution A for 1 minute (Polysciences, Warrington, PA).[9] Slides were evaluated for fungal elements at ×200, ×400, and ×630 with a Zeiss Axioskop microscope (Zeiss, Weesp, the Netherlands) using filter set 5. Branching, septate hyphae were considered to be morphologically compatible with but not proof of *Aspergillus* species.

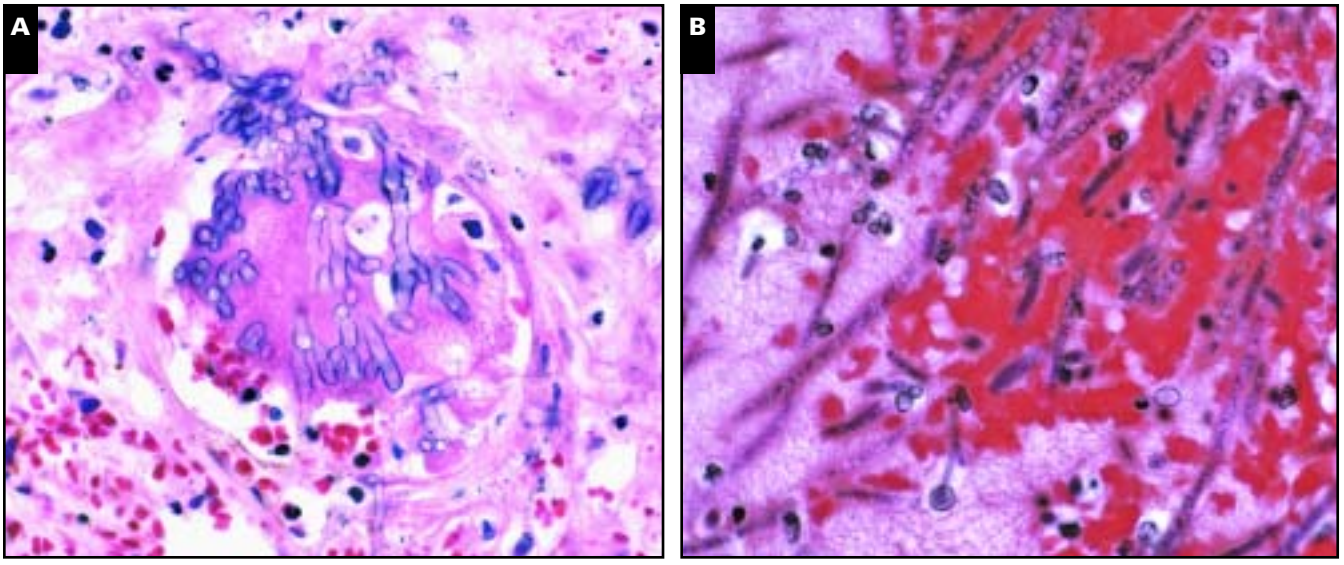

Image 1 Histopathologic features of invasive pulmonary aspergillosis in neutropenic patients. **A**, Angioinvasion. **B**, Intra-alveolar hemorrhage (**A** and **B**, H&E, original magnification ×400).

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Fungal cultures were processed by centrifuging 5 mL of BAL fluid for 10 minutes at 2,800*g*. Culture supernatant was removed, and the pellet was inoculated onto the following media: inhibitory mold agar (Hardy Diagnostics, Santa Maria, CA), Chromagar (Hardy), BCYE (Remel, Lenexa, KS), and brain-heart infusion agar with blood, chloramphenicol, and gentamicin (Remel). Lung biopsy specimens were plated as for BAL, but with the addition of Sabouraud dextrose agar (Remel) and without the Chromagar. Culture plates were incubated at 30°C in air and examined for 4 weeks.

All growth was evaluated for characteristic colonial morphologic features of *Aspergillus* species. Lactophenol cotton blue stains of material from colonies prepared by tease preparation or tape mount methods were examined microscopically. The microscopic features of conidiophores, vesicles, phialides, and conidia present in the genus *Aspergillus* were assessed for identification of species.[10]

Statistics

For comparison of categorical data, a 2-tailed Fisher exact test was used. A P value less than .05 was considered statistically significant. All analyses were performed using the Prism 3.0 statistical package (GraphPad, San Diego, CA).

Results

Patient Characteristics

The records of 61 patients with a pathologic diagnosis of invasive aspergillosis were retrieved from the database of the Pathology Department. Histopathologic material from autopsy or biopsy was available for 40 patients (66%).

The demographic features of the 40 cases studied are summarized in **Table 1**. Of the 40 patients, 10 belonged to

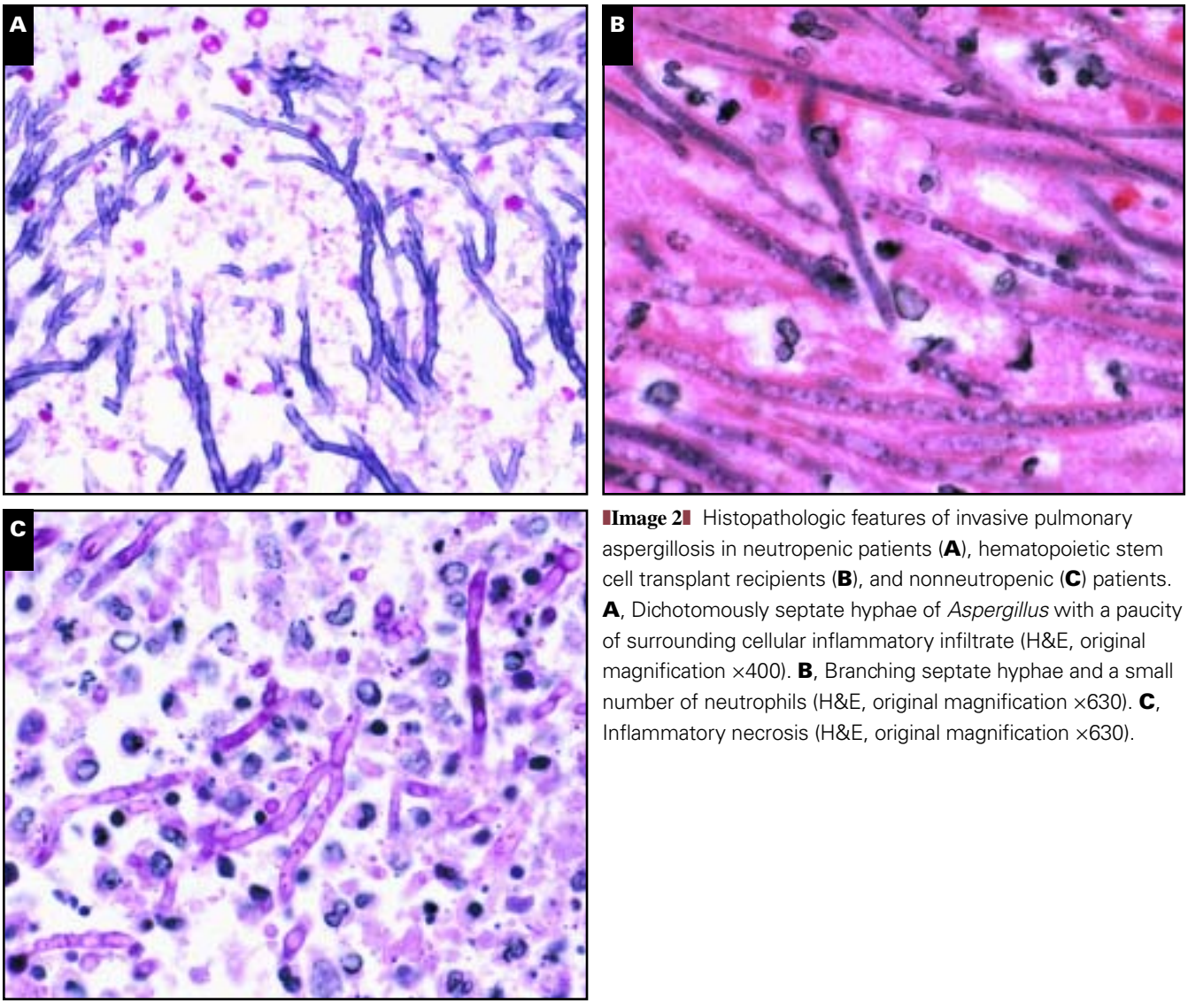

Image 2 Histopathologic features of invasive pulmonary aspergillosis in neutropenic patients (**A**), hematopoietic stem cell transplant recipients (**B**), and nonneutropenic (**C**) patients. **A**, Dichotomously septate hyphae of *Aspergillus* with a paucity of surrounding cellular inflammatory infiltrate (H&E, original magnification ×400). **B**, Branching septate hyphae and a small number of neutrophils (H&E, original magnification ×630). **C**, Inflammatory necrosis (H&E, original magnification ×630).

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the neutropenic cohort, neutropenia never developed in 20, and 10 were HSCT recipients. All HSCTs were allogeneic and were complicated by GVHD. The median time between stem cell infusion and tissue biopsy or autopsy was 95 days (range, 49-338 days).

Underlying Disease

In the neutropenic cohort, the most frequent underlying disease was a hematologic malignancy or other hematologic disorder (Table 1). In the nonneutropenic cohort, hematologic malignancies, inherited immunodeficiencies, and AIDS were the predominant underlying diseases. In the HSCT cohort, 90% of patients had malignancies, with only 1 recipient having had congenital immunodeficiency.

Microbiology and Patterns of Infection

From 6 neutropenic cases with positive cultures, *Aspergillus fumigatus* was isolated in 2 cases, *Aspergillus terreus* in 2 cases, *Aspergillus flavus* in 1 case, and an *Aspergillus* unidentified to the species level in 1 case. From 9 nonneutropenic cases with positive cultures, *A fumigatus* was the only species isolated in all cases. From 4 HSCT recipients with positive cultures, *A fumigatus* also was the most common *Aspergillus* species isolated (3 cases), followed by *Aspergillus niger* (1 case). Extrapulmonary aspergillosis was documented in 4 (40%), 6 (30%), and 3 (30%) of neutropenic, nonneutropenic, and HSCT cohorts, respectively.

Histopathologic Patterns

Two patterns of tissue injury by IPA were found in the 3 cohorts **Table 2**. The first pattern was characterized by angioinvasion, hemorrhagic infarction, and intra-alveolar hemorrhage. The second pattern was characterized by inflammatory necrosis and granulomas. Angioinvasion by *Aspergillus* hyphae was more prevalent in the neutropenic (6 [60%]) and HSCT (8 [80%]) cohorts than in the nonneutropenic cohort (4

Table 1
Characteristics of Neutropenic and Nonneutropenic Patients and HSCT Recipients With Invasive Pulmonary Aspergillosis Within 30 Days Before Autopsy or Biopsy*

Factor	Neutropenic Group† (n = 10)	Nonneutropenic Group (n = 20)	HSCT Recipients (n = 10)
Median age (range), y	29 (4–70)	43 (18–70)	49 (19–64)
Female/male	4/6	8/12	3/7
Underlying diseases			
Hematologic malignancies	4 (40)	4 (20)	6 (60)
Other hematologic disorders	4 (40)‡	1 (5)§	3 (30)
AIDS	2 (20)	6 (30)	0 (0)
Primary immunodeficiencies	0 (0)	6 (30)‖	1 (10)
Other	0 (0)	3 (15)	0 (0)
HSCT conditioning			
Myeloablative	—	—	6 (60)
Nonmyeloablative	—	—	4 (40)
Therapeutic agents			
Chemotherapy	4 (40)	2 (10)	0 (0)
Corticosteroids	3 (30)	10 (50)	9 (90)
Other immunosuppressive agents	3 (30)	1 (10)	9 (90)
Antifungal agents	8 (80)	14 (70)	7 (70)

HSCT, hematopoietic stem cell transplant.
* Data are given as number (percentage) unless otherwise indicated.
† Absolute neutrophil count <500/μL (<500 × 10^9/L) for more than 1 wk.
‡ Aplastic anemia (2), myelodysplasia (1), and Fanconi anemia (1).
§ Idiopathic CD4 lymphopenia.
‖ Chronic granulomatous disease (2), IgA deficiency (1), idiopathic CD4 lymphopenia (1), primary immunodeficiency (1), T–cell proliferative disorder (1).

Table 2
Histopathologic Pattern in Neutropenic and Nonneutropenic Patients and HSCT Recipients With Invasive Pulmonary Aspergillosis*

Group	Angioinvasion	Hemorrhagic Infarction	Intra–alveolar Hemorrhage	Coagulative Necrosis	Granulomas	Inflammatory Necrosis
Nonneutropenic (n = 20)	4 (20)	3 (15)	10 (50)	8 (40)	2 (10)	10 (50)
Neutropenic (n = 10)	6 (60)†	4 (40)	9 (90)†	3 (30)	0 (0)	1 (10)†
HSCT (n = 10)	8 (80)†	5 (50)	10 (100)†	7 (70)	0 (0)	2 (20)

HSCT, hematopoietic stem cell transplant.
* Data are given as number (percentage).
† $P < .05$ (Fisher exact test) was considered statistically significant in comparison with the nonneutropenic group.

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[20%]; $P = .045$ and $P = .0041$, respectively). Intra-alveolar hemorrhage also was observed more frequently in neutropenic patients (9 [90%]; $P = .048$) and in HSCT recipients (10 [100%]; $P = .011$; Image 1). In the lung tissues of neutropenic patients and HSCT recipients, a conspicuous paucity of neutrophilic and monocytic lesions was observed (Image 2). In the HSCT cohort, there also was a trend toward a higher frequency of coagulative necrosis (7 [70%]) than in the other cohorts (neutropenic cohort, 3 [30%]; nonneutropenic cohort, 8 [40%]). In nonneutropenic patients, inflammatory necrosis was observed as the predominant pattern of host response (10 [50%]; Image 2). Thus, the neutropenic and HSCT cohorts had a similar histologic pattern, whereas the nonneutropenic patients had a different pattern of infection and inflammation.

Discussion

The histopathologic patterns of lung tissues of neutropenic patients and HSCT recipients with IPA were similar and were characterized by angioinvasion and intra-alveolar hemorrhage. By comparison, in the nonneutropenic patients, the histologic pattern was clearly different, being characterized by inflammatory necrosis. To our knowledge, this is the first time that angioinvasion caused by *Aspergillus* species has been statistically associated with neutropenia in human tissue in relationship to the host responses.

It is indeed striking that even though HSCT recipients have normal peripheral blood neutrophil counts, there is no influx of neutrophils in the lung tissue. We speculate that this histologic model may reflect a defect in the recruitment and function of neutrophils after recognition of fungus-associated molecular patterns. Numeric recovery of neutrophils following HSCT does not necessarily mean recovery in function of neutrophils.[11] There is a reconstitution period for innate and adaptive immunity following HSCT infusion, during which cellularity does not correlate with recovery of cellular function.[11]

Studies from the last 2 decades have elucidated the recovery of innate and adaptive immunity following HSCT; however, the complete picture of immune system recovery remains incompletely defined, particularly regarding specific pathogens, such as *Aspergillus* species.[11] The period for recovery of numeric and functional capacity among different types of immune-mediating cells varies from a few weeks (eg, natural killer cells recovery) to at least 1 year (eg, T cell–dependent antibody production). The posttransplantation period of HSCT in our study is still within the recovery period of immune reconstitution, suggesting a continued immunoregulatory impairment in response to *Aspergillus* species.

The pattern of tissue injury in profoundly neutropenic patients in this study underscores the concept that neutrophils are an important line of defense against *Aspergillus*.[6] Without neutrophils, hyphae elongate and invade lung parenchyma and pulmonary blood vessels. The result is thrombosis of blood vessels, hemorrhagic infarction, and intra-alveolar hemorrhage. Perhaps the thrombosis of blood vessels can also be induced by the ability of *Aspergillus* to stimulate endothelial cells to become prothrombotic by expressing thromboplastin.[12] The same histologic model was found in animals with chemotherapy-induced neutropenia challenged with *A fumigatus* conidia. Berenguer et al[8] observed that angioinvasion and thrombosis often resulted in extensive pulmonary infarction and intra-alveolar hemorrhage in profoundly neutropenic rabbits beyond the focus of vascular invasion. Balloy et al[13] also demonstrated that in mice with chemotherapy-induced neutropenia, there was congestion within the alveoli but no inflammatory exudates involving neutrophils and monocytes.

The absence of neutrophils in a profoundly neutropenic host seems to have a permissive effect in allowing unchecked proliferation of hyphae through tissue planes, including the walls of blood vessels. This contrasts with nonneutropenic, non-HSCT hosts in whom angioinvasion is seldom observed, presumably because of a more intact innate immune host response to contain hyphal forms and in whom inflammatory necrosis predominates. This type of nonangioinvasive pulmonary lesion was also the hallmark of IPA in animals immunosuppressed with corticosteroids.[8,13,14]

The appearance of tissue injury in HSCT recipients with IPA is similar to that of neutropenic patients. All HSCT recipients had GVHD and were receiving corticosteroids and other immunosuppressive drugs. The time between transplantation and autopsy or biopsy did not seem to be a contributing factor for the histopathologic features. Furthermore, the type of the conditioning treatment (myeloablative or nonmyeloablative) did not seem to affect the histologic pattern. Shaukat et al[15] conducted a retrospective study of allogeneic HSCT recipients and also documented that the predominant lung histopathologic feature was acellular coagulative necrosis. Notably, hyphal angioinvasion was observed in some of these cases. Thus, HSCT recipients with IPA seem to have a functional neutropenia that permits *Aspergillus* to become angioinvasive, as in patients with true neutropenia.

Neutrophil chemotaxis to the site of infection in the lungs is orchestrated by a complex of secreted mediators, and receptors of innate and adaptive immunity can be affected. Among the chemokines that can have a critical role in neutrophil recruitment are interleukin (IL)-8 and macrophage inflammatory protein-1α.[6] Min et al[16] found that the level of IL-8 in the serum was elevated during the first week after the stem cell infusion compared with the concentration before the use of chemotherapy and did not show any statistically significant change after the second week. However, it is unknown whether the serum IL-8 concentration or the local production of IL-8 responds to a challenge of *Aspergillus* in the lung by

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alveolar macrophages of HSCT recipients. Because there may be no correlation between circulating cytokines and those observed in tissues and BAL fluid of mice infected with *A fumigatus*, impaired local production may have a role.[14] Macrophage inflammatory protein-1α has chemotactic effects on interferon γ–activated neutrophils.[17] The late recovery of CD4 T cells, which produce interferon γ,[11] may result in less influx of neutrophils.

The presence of GVHD can inhibit even further the normal reconstitution of pulmonary and systemic host defenses. GVHD has deleterious effects on T-cell recovery and function through production of immunosuppressive cytokines by regulatory cell populations (eg, transforming growth factor β and IL-10), through apoptosis, and through direct damage to the thymus.[11] Immunoregulatory cytokines, such as IL-10, can impair the secretion of proinflammatory cytokines tumor necrosis factor α (TNF-α), IL-1β, IL-6, and IL-12 and the protective cell-mediated immunity.[18] TNF-α is not directly chemotactic for neutrophils, but it probably contributes to the chemotaxis of neutrophils via secondary mechanisms, including induction of neutrophil-chemotactic chemokines, adhesion molecules, and neutrophil-independent pathways.[19] Duong et al[14] demonstrated that the time of release of IL-1β and IL-6 in lung homogenates of immunocompetent mice infected with *A fumigatus* correlated with the time of neutrophil infiltration in the lung tissue. Thus, low levels of TNF-α, IL-1β, and IL-6 may further impair the chemotaxis of neutrophils.

The histopathologic pattern observed in the neutropenic cohort correlates with the radiologic findings in this population. A most common early manifestation of IPA in neutropenic hosts is the halo sign,[20-26] which is seen as an area of ground-glass attenuation around a nodule of soft tissue attenuation. The ground-glass attenuation is the result of hemorrhage and/or edema, whereas the nodule is infarcted tissue by *Aspergillus* species. Our findings are consistent with those of the seminal radiopathologic study of Hruban et al.[22] However, we also observed areas of pulmonary edema and intra-alveolar hemorrhage beyond the region of coagulative necrosis that may explain the early reversible component of the halo sign during treatment.

It is also noteworthy that Kojima et al[27] found that HSCT recipients with IPA could develop a halo sign at any time during the phase of neutropenia, as well as several months after transplantation. This finding is consistent with the histopathologic pattern that we have described for this cohort of patients. The absence of neutrophils in the areas of lung tissue that has been invaded by *Aspergillus* species leads to a radiologic manifestation similar to that in patients with true neutropenia.

Although the lungs are the most frequent sites of infection caused by *Aspergillus* species in immunosuppressed patients, there are studies reporting that patients who died of or with IPA also had proven disseminated aspergillosis at autopsy.[1,28] Aspergillosis of the central nervous system (CNS) is a particularly devastating complication of disseminated infection. There were 13 cases of disseminated aspergillosis in this reported series. Of the 13 patients, 8 had CNS infection. From those 8 cases, we retrieved CNS specimens from 4 (1, 2, and 1 of the neutropenic, HSCT, and nonneutropenic cohorts, respectively) with CNS-disseminated aspergillosis. The lesions of the tissue injury from these 4 cases were consistent with those of IPA that were described for each cohort. However, the small number of cases cannot lead to a firm conclusion about the histopathologic pattern of CNS-disseminated aspergillosis.

Neutropenia and HSCT confer a similar pattern of predominantly angioinvasion and infarction, whereas nonneutropenic hosts have a predominant pattern of inflammatory necrosis with minimum angioinvasion. Our study also shows the importance of studying the kinetics of cytokine and chemokine production in different nonneutropenic patients with invasive aspergillosis to understand the mechanisms of impaired neutrophil recruitment and to design immune augmentation strategies tailored to specific patient groups.[29]

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