

Case Report

Usefulness of Fungiflora Y to detect fungus in a frozen section of allergic mucin

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Allergic fungal sinusitis is a non-invasive disease characterized by pansinusitis with nasal polyps, with most causative agents being members of the dematiaceous fungi. Reported herein is the ninth Japanese case of allergic fungal sinusitis; an *Alternaria* species was culturally identified as the etiological agent. The present patient, a 32-year-old man, presented with multiple sinusitis and nasal polyps. Allergic mucin, which is a histological hallmark of this disease, was examined intraoperatively on frozen section. Although characteristic histology, including laminated masses of mucin and aggregates of eosinophils, was seen in HE-stained sections, fungal hyphae were scarce and recognized merely as clear spaces. Because fluorescent stilbene derivatives seem useful for the rapid detection of fungi, Fungiflora Y, a commercially available staining kit that includes a fluorescent stilbene derivative, was applied to the sections, and hyphae were successfully visualized using a fluorescence microscope. This stain takes only several minutes to perform. It is concluded that Fungiflora Y should be used when fungal elements are hard to find on HE stain, especially in cases in which rapid detection of fungus is requested.

Key words: allergic sinusitis, fluorescent, Fungiflora Y, fungus

Fungal sinusitis is usually divided into four categories: (i) acute/fulminant (invasive), (ii) chronic/indolent (invasive), (iii) mycetoma/fungus ball, and (iv) allergic fungal sinusitis (AFS).^{1,2} AFS is a distinct clinicopathological entity characterized by a non-invasive condition, and it is the most common form out of these four categories.^{1–3} Type I and/or

type III hypersensitivity reactions to non-invasive fungal elements are likely to be important processes in this disease.^{4–6} AFS accounts for 6–8% of all chronic sinusitis requiring surgical intervention in the USA.^{3,7} In Japan, however, it is very rare, and only eight cases have been reported to date.⁸ Patients with AFS are usually immunocompetent atopic young adults with histories of recurrent sinusitis despite multiple surgical procedures.¹ Nasal polyposis, asthma, elevated total serum IgE levels, and fungus-specific IgE and IgG levels, as well as peripheral blood eosinophilia, are characteristic of AFS.¹

The diagnosis of AFS is primarily histopathological, and allergic mucin within sinuses serves as a histological hallmark of the disease.^{4,5} Fungal hyphae must be seen, but these are usually scarce in the allergic mucin.^{4,5,7} Given that fungal hyphae are scarce within allergic mucin, their identification is extremely difficult unless specific histological stains, such as PAS or Grocott's methenamine silver (GMS), are used. However, structures other than fungi within the sections are also stained by these two methods. Fluorescent stilbene derivatives afford a solution to this problem. They non-specifically bind with a high affinity to cellulose, chitin and other β -linked polysaccharide polymers, which are components of fungal cell walls.^{9–12} A large variety of fungi are stained selectively, yielding an intense fluorescence under a fluorescence microscope.^{9–12} Several stilbene derivatives, such as Calcofluor white, Blankophor BA and Uvitex 2B, have been described in previous reports.^{9–12} Fungiflora Y (Alfresa Pharma, Osaka, Japan) is a commercially available kit that consists of two different solutions: a kind of hematoxylin and a fluorescent stilbene derivative. Information regarding the precise chemical details of this stilbene derivative has not been announced publicly. We applied Fungiflora Y to the intraoperative examination of frozen sections of allergic mucin from patients with AFS, and describe its usefulness in the rapid detection of fungal hyphae.

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Figure 1 (a) Coronal CT showing that the right ethmoid sinus is filled with a soft-tissue density mass extending into the right maxillary sinus. (b) Axial CT of the mass, which extends from the right ethmoid sinus to the sphenoid sinus. There is no bony wall destruction.

CLINICAL SUMMARY

A 32-year-old man with a history of seasonal allergic rhinitis and who had had frontal headaches and nasal obstruction for 10 years, began complaining of worsening headaches several months before his admission. He was referred to an otorhinolaryngologist who noted right nasal polyps. His initial CT scan demonstrated that his right ethmoid sinus, right sphenoid sinus and frontal sinus were filled with soft-tissue-density material extending to the right maxillary sinus (Fig. 1). There was no bony erosion. The low density of the inner surface of the sinuses indicated mucosal edema. Laboratory findings showed an elevated total eosinophil count in his peripheral blood (white blood cell count, 6400/ μ L; neutro-

phils, 3808/ μ L; eosinophils, 371/ μ L; lymphocytes, 1805/ μ L; monocytes, 377/ μ L). His serum level of IgE (1542 IU/mL; reference range, <600 IU/mL) was elevated. Clinically, papillomatous tumor or fungal infection was suspected. The patient underwent right nasal polypectomy and endoscopic endonasal sinus surgery, including right maxillotomy and right ethmoidectomy. A whitish-green inspissated mucoid material was noted in the right ethmoid and maxillary sinuses. The inspissated material from the ethmoid sinus was intraoperatively submitted to the pathology laboratory at Kinki University for frozen section examination because the physicians were not certain what kind of sinus lesion they represented. Rapid diagnosis of sinus fungal disease was made on routine HE staining and a fluorescence microscopic study using Fungiflora Y. Further operation could not be performed due to regional bleeding.

PATHOLOGICAL FINDINGS

The pathological specimen submitted for frozen section examination was a $4 \times 4 \times 3$ mm whitish friable tissue. Microscopically it demonstrated laminated masses of mucin-containing aggregates of degenerated eosinophils with pyknotic nuclei and dispersed granules, and cellular debris, including eosinophilic granules and a few Charcot-Leyden crystals (Fig. 2a). Fungi were identified with difficulty as clear spaces on HE-stained sections. Their precise morphology was obscure. For non-specific fluorescent staining, Fungiflora Y was applied to an adjacent section from the same consecutive series including an HE-stained section. Frozen sections were stained with a few drops of Fungiflora Y solution A (a kind of hematoxylin) for 1 min, rinsed with tap water for 2 min, stained with solution B (fluorescent stilbene derivative) for 2 min, rinsed with tap water and mounted with Entellan New (Merck, Darmstadt, Germany) after dehydration through ethanol and xylene. Slides were observed using a fluorescence microscope (Nikon Eclipse E800, Nikon, Tokyo, Japan) equipped with a filter module UV-1 A (excitation, 365 nm; dichroic filter, 400 nm; barrier filter, 400 nm) and a mercury lamp for illumination. Fungal hyphae fluoresced apple-green, were sharply delineated, and contrasted well against the dark background (Fig. 2b). Ethmoid tissues submitted later were processed routinely into paraffin sections. These sections demonstrated almost the same histology as the frozen sections. In addition, several pieces of mucosal tissue were found in the mucin, and these were infiltrated with eosinophils. There was no evidence of tissue invasion by the fungus into the mucosa. GMS staining showed distinct septate short hyphae with thick cell walls, appearing to branch at acute angles (Fig. 3).

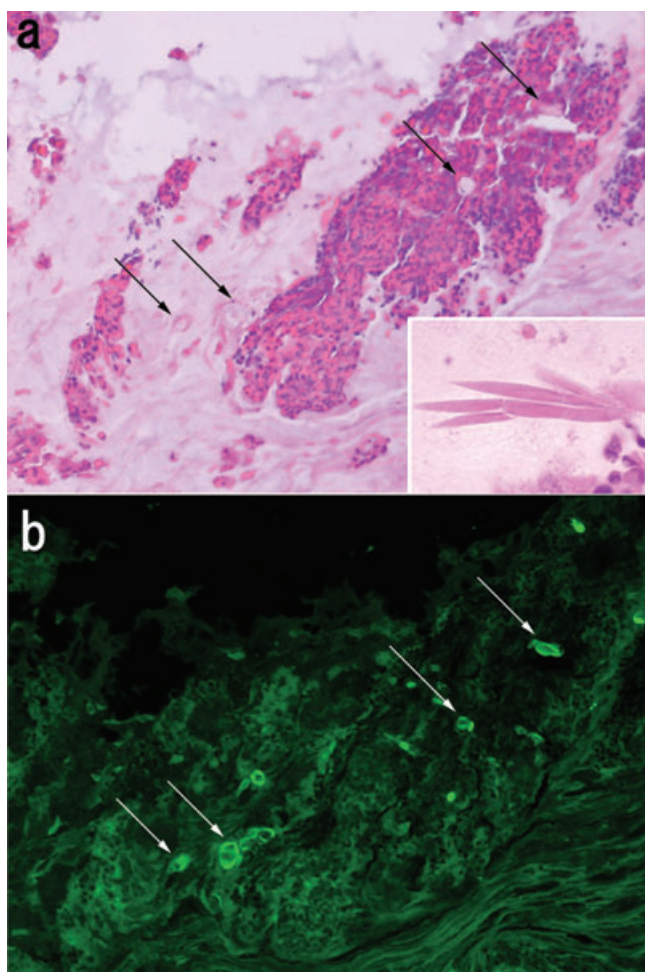


Figure 2 (a) Allergic mucin in a frozen section. Accumulations of eosinophils embedded in mucin are seen. Fungal hyphae fail to be stained with HE, and are indicated only by their resulting negative image against an otherwise stained background (arrows). Inset: Charcot-Leyden crystals are eosinophilic and characterized by a spindle shape with sharply pointed ends. (b) The adjacent section to that shown in (a), stained with Fungiflora Y and observed under a fluorescence microscope. Short hyphae fluoresce an intense apple green. The fluorescing hyphae indicated by the arrow are almost identical to the uncolored hyphae indicated by the arrow in (a).

MYCOLOGY

Submitted fungal cultures grew rapidly on potato dextrose agar at 25°C, attaining a diameter of 4.2 cm within 7 days, becoming gray to olivish black and felty. The conidiophores were smooth, brown, simple or branched, straight or flexuous. Conidia were muriform, with a short cylindrical beak, 18–35 × 10–12 µm in size, oblong to pyriform in shape, pale to golden brown, smooth or verrucose (Fig. 4). Isolates from the patient were identified as *Alternaria* species.

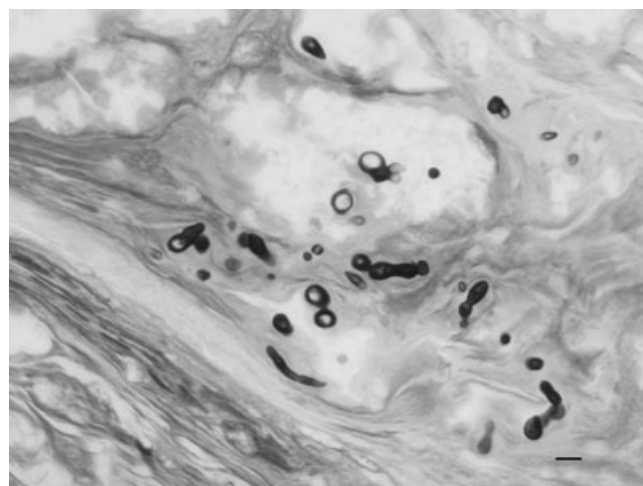


Figure 3 Grocott's methenamine silver-stained paraffin sections. Septate fungal hyphae are clearly visible. Bar, 10 µm.



Figure 4 Culture isolates were identified as *Alternaria* species. Note the branched acropetal chains and multicelled, obclavate to obpyriform conidia with conical beaks. Bar, 50 µm.

DISCUSSION

In 1983 Katzenstein *et al.* retrospectively reviewed microscopic slides of 119 specimens from chronic sinusitis surgery and found nine specimens histologically indistinguishable from mucoid impaction of the bronchi observed in patients with allergic bronchopulmonary aspergillosis (ABPA).^{13,14} Seven of the nine specimens were noted to have fungal hyphae resembling *Aspergillus* species within them. These authors presented evidence that clinical and pathological findings in these seven cases comprised a distinct entity possibly analogous to ABPA, but occurring in the paranasal sinuses, and they proposed the name allergic *Aspergillus* sinusitis. After that, similar cases of fungal culture yielding fungi other than *Aspergillus* species have been reported, and

the condition is now named AFS.^{6,15–17} AFS is a common fungal sinusitis in the English-language literature but it is very rare in Japan and only eight cases have been reported to date.⁸ The present case is the ninth Japanese case. Members of the dematiaceous fungi are the most common fungi implicated in AFS.^{6,16} Among them, *Bipolaris* species is the most common. *Alternaria* species, which was cultured in the present case, is one of the more common dematiaceous fungi in AFS.

The exact mechanism underlying the pathogenesis of AFS is not well understood but it is considered to be similar to that of ABPA, involving both type I and type III hypersensitivity reactions.^{1,4–6} Trapping of inhaled fungal antigen within a sinus already partially obstructed due to other inhalant allergies is likely to lead to early and late-phase allergic inflammation. Recruitment of activated eosinophils and the release of subsequent mediators, such as eosinophilic cationic protein and eosinophilic granular major basic protein, cause epithelial damage and mucosal edema, leading to further sinus obstruction and accumulation of allergic mucin.^{6,18,19} Although it appears clear that eosinophils play an important role in the development of AFS, the factors that trigger eosinophilic inflammation remain to be determined. Initiation of the inflammatory cascade leading to AFS appears to be a multifactorial event, involving IgE-mediated sensitivity, specific T-cell human leukocyte antigen receptor expression, exposure to specific fungi, and aberration of local mucosal defense mechanisms.^{4,5}

The definitive histopathology of AFS is extramucosal inspissate of allergic mucin within sinuses, which is identical to the material seen in the bronchi of ABPA patients.^{13,14} The diagnosis of AFS is primarily histopathological and allergic mucin serves as a hallmark of the disease.^{4,5,7} HE staining of the allergic mucin shows a pale eosinophilic or basophilic amorphous layer of mucous with sheets of degenerated eosinophils and Charcot–Leyden crystals.^{7,13,14} Fungal hyphae must be found within allergic mucin, but they are sporadic. Furthermore, they are difficult to visualize on HE stain, and are mostly identified as uncolored clear spaces.^{2,7,16} Therefore, their identification within the mucin is extremely difficult unless specific histological stains are used. Although GMS can distinctly visualize fungal elements, it may be useless for intraoperative frozen section examination because of its long staining time, which results in a delay in reporting. In contrast, staining with fluorescent stilbene derivatives was described to be an accurate and rapid method for the detection of fungal elements.²⁰ When viewed with a properly equipped fluorescence microscope, fungal elements fluoresce an intense apple green.^{9–12} We applied Fungiflora Y, a commercially available kit that includes a fluorescent stilbene derivative, to the intraoperative frozen section examination of allergic mucin from a patient with AFS. It was an extremely fast and simple method resulting in easy identification of

fungi, which was suitable for intraoperative examination. A fluorescence microscope is the only special item of equipment required. Rapid recognition of fungal elements within allergic mucin can reassure physicians of the absence of both invasive fungal disease and a neoplastic condition during operations. This avoids radical debridement and aggressive surgery. Prompt endoscopic sinus surgery with complete removal of impacted fungal debris and ventilation of the involved sinuses can then be instituted soon after frozen section examination. Fungiflora Y is applicable to formalin-fixed, paraffin-embedded specimens.¹⁰ Furthermore, there is no difference in staining results between frozen sections and paraffin sections. If an antibody against fluorescent stilbene derivatives is available, immunohistochemical detection of fungi can be performed. A fluorescence microscope is not necessary in such cases. It is concluded that Fungiflora Y should be used for the intraoperative examination of a specimen suspected of fungal etiology.

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