

Detection of Fungi in Sinus Fluid of Patients with Allergic Fungal Rhinosinusitis

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We aim to develop a rapid, accurate and sensitive diagnostic assay with which to detect the surface antigens of fungi thought to be involved in allergic fungal rhinosinusitis (AFRS), by assessing the usefulness of immunofluorescence microscopy (IMF) and enzyme linked immuno-absorbent assays (ELISA). The age, sex, clinical symptoms and signs, imaging (CT and/or MRI), microbiological subculture data, sinus contents, blood eosinophilia, aspergillosis precipitins, radioallergoabsorbent technology (RAST) for fungal allergens and histopathology were performed on individuals undergoing endoscopic sinus surgery for suspected AFRS. Thirteen patients were examined, and five monoclonal antibodies raised to the surface washings of various fungi were found to recognize and differentiate between fungal species implicated in sinus disease, i.e. *Aspergillus niger*, *Alternaria alternata*, *Cochliobolus lunata*, *Penicillium expansum* and *Cladosporium* species. The IMF microscopy proved to be a useful assay to distinguish visually between the cultured fungi, but was less useful for visualization of fungi in the patient samples. However, ELISA assays with 5 monoclonal antibodies gave clear and unambiguous data as to the presence of certain fungi within the patient samples. There is good correlation between the ELISA data and the pathology findings. This preliminary study suggests that both IMF and ELISA techniques may offer an important advance in this area. **Key words:** allergic fungal rhinosinusitis, enzyme linked immunosorbent assay, immunofluorescence microscopy, monoclonal antibodies.

INTRODUCTION

Fungal disease of the paranasal sinus was once considered to be rare. Over the last decade, however, the number of diagnosed cases has risen dramatically and there are now a number of recognized categories of non-invasive and invasive paranasal sinus disease (Table I).

Whilst it is now widely accepted that allergic fungal rhinosinusitis (AFRS) is a distinct clinical problem, controversies surrounding its differential diagnosis and the best treatment regime for the patient continue. Allergic fungal rhinosinusitis, first described in 1983 (2), was initially termed “allergic aspergillus sinusitis” because of its histopathological similarity to allergic bronchopulmonary aspergillosis. Subsequent studies, however, have implicated the involvement of several other fungi in this disease such as *Bipolaris*, *Alternaria* and *Curvularia* (3–5). It typically occurs in atopic immunocompetent young adults, but it is also seen in other age groups (rarely below 10 years). It is often associated with nasal polyps and with asthma, but it can also be seen, albeit rarely, in association with aspirin intolerance. The patient usually has a long history of chronic rhinosinusitis, which has proved unresponsive to antibiotics (6, 7). AFRS now accounts for up to 8% of all chronic rhinosinusitis requiring surgical intervention.

The true aetiology of AFRS remains obscure: it is difficult to diagnose as there are no universally accepted standards described in the literature. To date, the otorhinolaryngologist has had to rely on a medley

of non-specific symptoms and pathological, radiographic and surgical clues (type 1 hypersensitivity, asthma, nasal polyps, unilateral or bilateral CT signs including bone erosion, eosinophilic mucus without fungal invasion and eosinophilia, presence of fungal hyphae shown by cytological staining and axenic culture), which suggest the presence of AFRS. Perhaps the most widely accepted hallmark of AFRS is the allergic mucin, which is characterized by eosinophilia and the presence of Charcot-Leyden crystals (5, 6). Furthermore, fungal hyphae can be cytologically stained with Gomori's methamine silver stain (6).

Just as there is no clear consensus on the criteria with which to diagnose AFRS, so too there is some disagreement regarding its treatment. Indeed, many patients undergo multiple procedures and treatments (surgery and medication) in an effort to suppress and cure this chronically remitting and relapsing condition. After diagnosis, the cornerstone in the management of AFRS is the surgical ventilation and drainage of the affected sinus with removal of all fungal material and allergic mucin, followed by a regime of nasal irrigation and endoscopic debridement, coupled with the use of topical or systemic corticosteroids. Opinion is deeply divided as to the efficacy of additionally treating the patient with systemic antifungal drugs (8). A new horizon in the treatment of AFRS may be desensitization immunotherapy, whence the patients are treated with the relevant fungal antigens to alleviate crust formation and polyp recurrence (9, 10). However, such an

approach demands the accurate diagnosis of disease and a comprehensive survey of the fungi putatively involved in AFRS.

The literature concerning the fungi implicated in AFRS is confused. The fungi include both dematiaceous (darkly-pigmented) and non-dematiaceous members (3–5). Limited laboratory studies suggest that the most common fungus to be cultured from patient samples with AFRS is *Bipolaris* (*Dreschlera*) *spicifera*, but other fungi have also been implicated, particularly *Aspergillus* spp (*A. niger*, *A. fumigatus*, *A. flavus*), and *Curvularia lunata*, *Alternaria spicifera*, *Cladosporium herbarum*, *Exserophilum rostratum*, *Penicillium chrysogenum*. However, the detection of fungi that cause, or are associated with AFRS by classical methods, such as axenic culture by plating out on growth media, is subject to several limitations. These include the requirement for laboratory facilities, mycological expertise and the need for specimens to remain viable from collection to plating out. The method is slow, and may take several days for fungal growth and sporulation, which is itself currently a necessary marker for species identification. Such problems have severely restricted our knowledge with regards to the importance and distribution of fungi in AFRS. There is therefore a need for a rapid and sensitive assay that can easily be replicated with limited expertise and facilities that will enable patient treatment to be hastened (11).

At the University of Oxford we have the largest known collection of monoclonal antibodies (MAbs) raised to fungi of various species (c. 90, raised by FMD) and, amongst these are some pertinent to this proposed study. Our aim was to develop an antibody-based method for the rapid and accurate detection of fungi involved in AFRS. Such a rapid diagnosis assay would allow the confirmation of a clinical diagnosis and the initiation of early treatment, which, in turn would limit the potential for orbital and intracranial complications of the more serious invasive form of fungal rhinosinusitis.

Table I. Classification of fungal rhinosinusitis

Non-invasive	Invasive
Fungal ball (mycetoma)	Acute fulminant
Allergic fungal infection	Chronic—immunocompetent —immunocompromised Sclerosing Granulomatous (1)

MATERIALS AND METHODS

Patient samples and physical examination

The sinus contents of 13 patients (9 men and 4 women, age range 13–67 years) with persistent sinus problems strongly suggestive of fungus were removed by endoscopic sinus surgery. Samples were preserved by addition of equal amounts of phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.0115% Na₂HPO₄, 0.025% KH₂PO₄ at pH 7.2) containing 0.02% sodium azide and stored at 4°C.

A questionnaire was completed for each patient, denoting age and sex, symptoms and their duration, the results of medical examination, computed tomography (CT) scan and magnetic resonance imaging (MRI) data, microbiological subculture results, sinus contents analysis, occurrence of eosinophilia, presence of aspergillosis precipitins, the results of radioallergoabsorbent technology (RAST) for fungal allergens and details of treatment (Table II).

Monoclonal antibodies

Ten hybridoma cell lines producing monoclonal antibodies, used for the immunodetection of fungi in sinus fluid, were screened against a range of fungi and bacteria (details are given in Table III).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were performed according to the plate-trapped antigen method (PTA-ELISA), described by Karpovich-Tate et al. (12) Microtitre wells, arranged in strips (Lab. Systems, Helsinki, Finland, product no. 950.2037), were coated with either surface washings from solid cultures of various fungi and bacteria, fungal spore suspensions, or with patient's sinus fluid and they were incubated overnight at either 4°C or, in the case of fungal spores, at room temperature to allow germination to take place. Patient samples were vortexed for 5 min before plating out into micro-titre wells and were serially diluted into further PBS to give a dilution series in the range of 1 in 2 (v/v) to 1 in 256 (v/v). After immobilization of the antigen, plates were washed 3 times in PBS and dried for 20 min in a laminar flow hood and then blocked with 0.3% (w/v) casein in PBS. The wells were incubated successively with hybridoma cell lines containing monoclonal antibodies (MAbs) or for negative control with hybridoma cell culture fluid RPMI (Sigma R-7880) plus 5% foetal bovine serum for 1 h and then with either goat anti-mouse (Sigma A-0412) or anti-rat (Sigma F-1763) polyvalent immunoglobulin (classes IgG, IgA and IgM) peroxidase conjugate, diluted 1 in 1000 (v/v) in PBST (PBS plus 0.5% (w/w) Tween 20), for 1 h at room temperature. Between incubations the wells

Table II. Details of the patient questionnaire data, microbiological results and the microscopy and IMF findings from the patient sinus samples.

Sample no.	Age	Sex	Royal National Throat, Nose & Ear Hospital	University of Oxford	
			Microbiology	RAST for fungal allergens	Microscopy/IMF
1	67	M	Traces of <i>A. niger</i> , positive skin prick for <i>Aspergillus</i> and <i>Cladosporium</i>	No data	Spores of <i>Alternaria</i> , branching mycelium UA-PC3 +ve
2	18	M	<i>Cochliobolus</i> , blood	Negative for <i>Aspergillus</i>	Numerous fungal hyphae UA-PC3 +ve
3	13	M	Possible fungal content	Negative for <i>A. fumigatus</i> , <i>Cladosporium</i> and <i>Alternaria</i>	Cluster of <i>Alternaria</i> -like spores, few hyphae Autofluorescence
4	44	F	No data	Negative for <i>A. fumigatus</i>	<i>Alternaria</i> -like spores, no visible mycelium
5	39	M	No data	Positive for <i>A. fumigatus</i>	Numerous fungal hyphae BC-KH4 +ve
6	53	M	<i>A. fumigatus</i>	Negative for <i>A. fumigatus</i>	Fragments of mycelium BC-KH4 +ve
7	28	M	No data	Negative	No fungal structures
8	51	F	<i>S. aureus</i>	Negative	No fungal structures
9	36	M	<i>S. aureus S. pneumoniae</i>	Not done SPT for all fungi +ve	Spores of <i>Ulocladium</i>
10	34	M	<i>A. fumigatus</i> , Blood	Negative	<i>Cladosporium Aspergillus</i>
11	33	M	<i>A. flavus</i>	No data	Difficulty in examination of the sample—very viscous Abundance of hyphae— <i>Aspergillus</i> -like hyphae AF-CA2 +ve BC-KH4 +ve
12	37	M	No data	Grade 4 +ve for <i>A. fumigatus</i>	Abundance of <i>Aspergillus</i> -like hyphae AF-CA2 +ve BC-KH4 +ve
13	62	F	<i>S. aureus S. pneumoniae</i>	No data	Negative

were rinsed three times with PBST. The bound antibody was “visualized” by incubating wells with tetramethyl benzidine substrate solution for 30 min. The reaction was stopped by adding 50 μ l 3M H₂SO₄ and absorbance values were read on an ELISA automated microplate reader (Dynatech MRX, λ = 450 nm). All working volumes were 50 μ l per well, except for the blocking step, which was 100 μ l, and all ELISA incubations were performed at room temperature (Figs 1A, B and C).

Immunofluorescence microscopy with fungal spores

Fungal spores germinated on multi-well glass slides were air-dried and immunolabelled by successively incubating with 0.3% casein in PBS for 30 min, 1 h with primary MAb and another hour with an appropriate secondary goat anti-mouse fluorescein isothiocyanate conjugate (FITC) (Sigma F-9006), diluted 1 in 40 v/v in PBST or goat anti-rat γ -chain specific rhodamine conjugate (TRITC) (Southern Biotechnology, 3030-03), diluted 1/25 in PBST. Between each incubation step the slides were washed 3 times for 5 minutes with PBST. After the final wash, slides were

mounted in Citifluor/PBS glycerol (Citifluor UKC Chem. Lab., Canterbury, UK) and viewed with Olympus microscope using both white light with DIC optics and UV fluorescence optics with the following filters: excitation 535 nm/emission 610 nm, for TRITC detection, excitation 450–490 nm/emission 515–560 nm for FITC detection and excitation 340–380 nm/emission 430 nm for autofluorescence only. Photomicrographs were taken on Fuji Provia ASA 400 Daylight film.

Immunofluorescence microscopy with patients samples

Patient samples were vortexed for 5 min and 0.5 ml aliquots of each sample was transferred to an Eppendorf tube and all tubes were centrifuged for 5 min at 12,000 g and the supernatant was discarded. Pellets were re-suspended in 200 μ l goat serum (blocking step to stop non-specific binding) followed by incubation with the primary MAb (100 μ l) and anti-mouse FITC (100 μ l) or anti-rat TRITC (100 μ l) conjugate for 1 h, diluted in PBS (as above) and washed between incubations by alternately spinning and re-suspending three times in 1ml PBST for 5 min. The final

pellet was re-suspended in 100 μ l Citifluor, mounted on a glass slide and viewed by microscopy, as described previously.

Preparation of surface washings of fungal and bacterial cultures for ELISA tests

Surface antigens of fungi and bacteria, grown on potato-dextrose agar for 3–5 days were removed by washing the slant cultures with 5 ml sterile PBS and gently scraping the surface with a sterile pipette. Washing fluid was removed by suction, filtered through a 70 μ m pore size sterile nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove mycelial fragments, and then centrifuged for 5 min at 12,000 g to remove any fungal spores. The resulting supernatant was diluted 1 in 5 (v/v) into PBS and used to coat micro-titre plate wells, 50 μ l per well, for tests.

Preparation of fungal spore suspensions

Spores from fungi grown on potato-dextrose agar for 5 days, were dislodged and harvested by adding 5 ml of sterile distilled water and gently scraping the surface with a sterile pipette. The resulting spore suspension was removed by suction and filtered through a sterile nylon cell strainer as above to remove mycelial fragments. The spore concentration was adjusted to 5×10^5 spores per ml in 0.5% (w/v) glucose and dispensed into wells of a multiwell Teflon-coated glass slide (ICN, 6040805), 20 μ l per well for testing by immunofluorescence (IMF). For ELISA tests spore suspensions were plated into micro-titre wells, 50 μ l per well and serially diluted into further PBS. To allow spore germination, both glass slides and microtitre wells were incubated overnight in a sealed Petri dish with moist filter paper or in a polythene bag (respectively) at 24°C or at 37°C for *Aspergillus* spp.

RESULTS

Specificity of monoclonal antibodies

Of the 10 MABs tested against fungi grown *in vitro*, five showed potentially useful specificities in that they recognized surface washings of cultured fungi by ELISA and germinating spores by IMF. These were the antibodies AF-CA2, AF-DA10, BC-KH4, OX-CH1 and UA-PC3 (Table III, Figs 1A, B and C). Species of *Aspergillus* and *Penicillium* were specifically recognized by AF-CA2 by both IMF and ELISA. *Cochliobolus* was recognized by UA-PC3 and BC-KH4 by ELISA and IMF. *Cladosporium* was recognized by OX-CH1 by both ELISA and IMF. Mab UA-PC3 proved useful for the detection of other fungi implicated in AFRS, notably, *Alternaria*. None of the chosen MABs gave a significant cross-reaction with the bacteria tested (data not shown).

Microscopy of immunolabelled patient samples

Fungal structures, such as fragments of mycelium and spores, were clearly seen in some of the sinus fluid samples under the white light using Nomarski optics (Table II). Melanized spores of dematiaceous fungi such as *Alternaria* (Fig. 2, patient 1) and *Ulocladium* that can easily be identified by their morphology were found either singly or in clusters. Fragments of branched hyaline (non-pigmented) mycelium were also seen with Nomarski optics in patient sample 2 (Fig. 3A) and, in some cases, these hyphae show strong autofluorescence when viewed under UV light (Fig. 3B).

In immunolabelled samples there was considerable autofluorescence of background material when viewed under UV light with a blue filter (Fig. 4, patient 12). This background was considerably reduced by the use of goat antiserum as a blocker. Immunofluorescently labelled hyphae were seen in all samples except patients 7, 8 and 13 as seen for

Table III. Specificities of monoclonal antibodies tested by immunofluorescence with different fungi. –ve: negative results, no immunofluorescence; + + + + +: denotes level of immunofluorescence: (a) immunofluorescence with both non-germinated and germinated spores and mycelium; (b) immunofluorescence with only germinated spores and mycelium, but not non-germinated spores; (c) immunofluorescence with only parts of mycelium and germ tubes; (d) melanized spores are not immunolabelled, only germ tubes and mycelium

Fungi MABs	<i>Aspergillus</i>		<i>Penicillium</i> <i>expansum</i>	Dematiaceous fungi		
	<i>niger</i>	<i>flavus</i>		<i>Alternaria</i>	<i>Cochliobolus</i>	<i>Cladosporium</i>
AF-DA10	–ve	–ve	+	–ve	–ve	–ve
AF-CA2	+ + + + + a	+ + + + + a	+ + + + + b	–ve	–ve	–ve
BC-KH4	+ + + + + a	+ + + + + a	–ve	–ve	+ + c	+ + + a
UA-PC3	–ve	–ve	–ve	+ + + + + d	+ + + + + d	–ve
OX-CH1	–ve	–ve	–ve	–ve	–ve	+ + + + + b

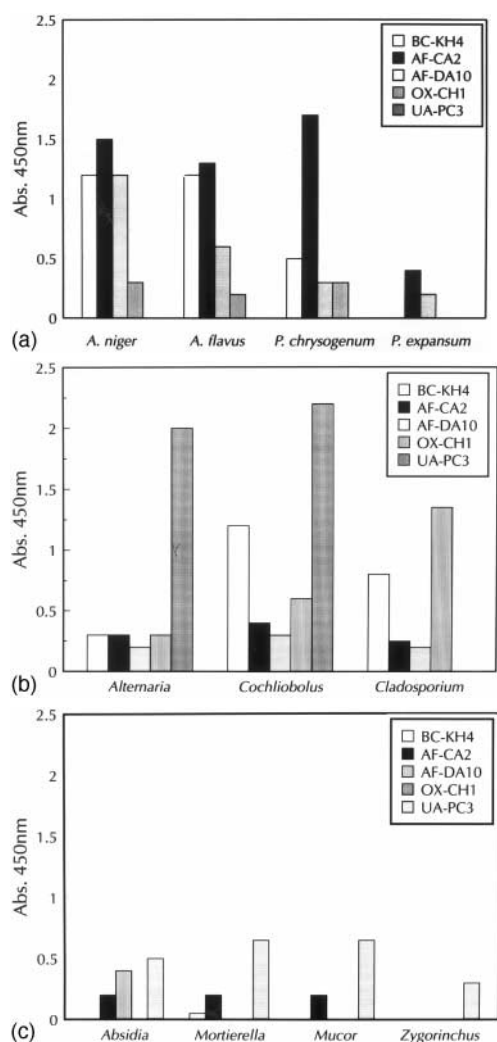


Fig. 1. (A, B and C) Absorbance values from PTA-ELISA tests with water-soluble surface antigens from cultures of various fungi implicated in AFRS with selected monoclonal antibodies.

example, with MAb BC-KH4 (Fig. 5) in patient sample 11. However, melanized spores and hyphae, such as *Cochliobolus*, did not become immuno-labelled.

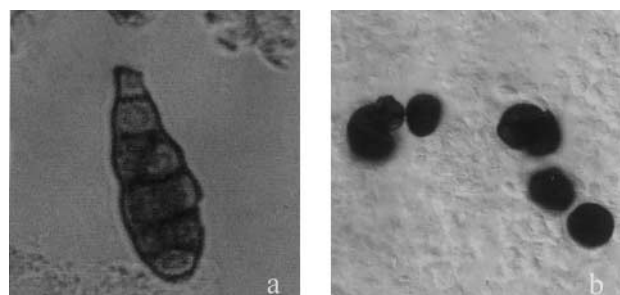


Fig. 2. White light Nomarski optics of patient sample 1 revealing melanized *Alternaria* spores.

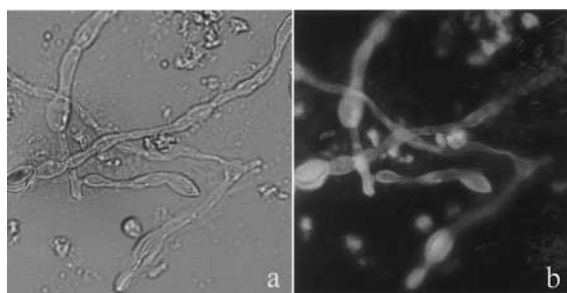


Fig. 3. (A) White light Nomarski optics of patient sample 2 and corresponding (B) UV image ($\times 40$).

ELISA results

All samples, except from patient 7, gave high absorbance values when tested with the rat MAb UA-PC3 and the murine MAb AF-DA10 and OX-CH1 but not with the anti-mouse peroxidase conjugate. High absorbance values were also obtained with the Sigma anti-rat peroxidase conjugate alone. Use of different blocking compounds, including goat serum, to reduce non-specific binding of a secondary anti-rat peroxidase conjugate prior to exposure of coated plates to the conjugate, failed to reduce the high absorbance values. A similar pattern of high absorbance values also occurred with the murine monoclonal antibodies AF-DA10 and OX-CH1.

DISCUSSION

The detection of fungal propagules in body fluids and tissues is fraught with problems. Current detection protocols rely heavily on culture methods, which can



Fig. 4. Intense autofluorescence masking image of patient sample 12 ($\times 40$).

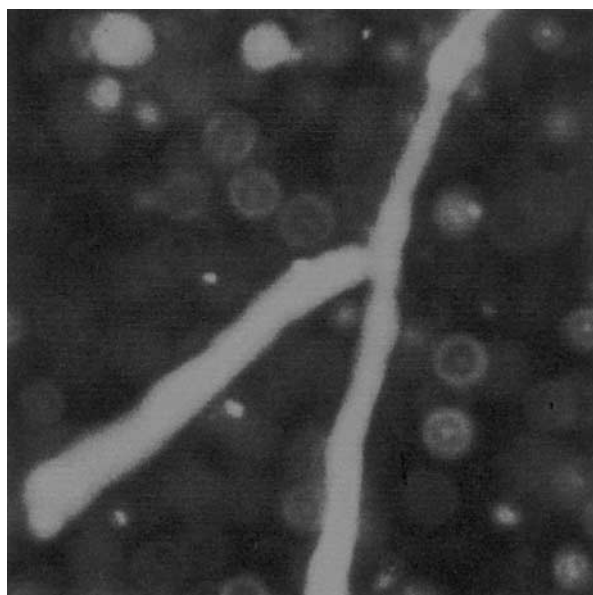


Fig. 5. Immunofluorescent image of patient sample 11 labelled with BC-KH4 ($\times 40$).

be slow, subject to contamination or indeed to failure of the fungus to grow and sporulate. In this study, and indeed in the immunofluorescent detection of fungi in ears (11), we have found that microscopy, coupled with the screening of patient samples immunolabelled with fungal specific monoclonal-antibodies has elegantly demonstrated the presence of fungi. The detection of hyphal fragments in patient samples by Nomarski optics alone is difficult, but the propagules are clearly visible when immunolabelled. The presence of *Alternaria*, *Ulocladium* and *Epicoccum*-like spores in the sinus contents from four patients was clearly and simply demonstrated by light microscopy of pelleted samples mounted in a glycerol-based liquid. Failure of these structures to become immunolabelled is not surprising because their outer surfaces are heavily melanized, preventing antibodies from penetrating and binding. The presence of such large melanized spores in sinus fluid is remarkable. It is not known whether they were derived from the outside through the nasal passages or whether they were a product of growth of the fungus in the sinus fluid. Paucity of fungal hyphae in some, but not all, specimens suggests the former.

Results of ELISA tests with patient samples were not definitive. Lack of truly negative sample made interpretation of these results difficult. Most puzzling was the high absorbance values obtained using anti-rat peroxidase conjugate alone with all patient samples except patient 1G who had no laboratory evidence of fungal infections. This effect was not

seen when the conjugate was tested against fungal antigens produced *in vitro*, even in a special sinus simulated fluid containing mucin (data not presented). These factors combined with the fact that the high absorbance values were not reduced by extensive blocking suggest that the Sigma anti-rat conjugate recognizes some compound in human sinus fluid, related directly or indirectly to fungal infection. This interesting observation should be pursued to identify the compound recognized and determine whether the Sigma anti-rat conjugate can be used in immunoassays as a quick screening method for the detection of fungal infections in sinus fluid.

A high degree of clinical suspicion has not always been rewarded by positive diagnostic histology and culture in allergic fungal disease. We have demonstrated the usefulness of IMF microscopy in the rapid detection of fungi, which may prove a useful tool in the future management of this fascinating condition.

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