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Diagnosis of invasive fungal infections in hematology and oncology Guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO)

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Abstract Invasive fungal infections are a primary cause of morbidity and mortality in patients with hematological malignancies. Establishing a definite diagnosis of invasive fungal infection in febrile neutropenic patients is particularly challenging and time-consuming, but a delay of antifungal treatment leads to higher mortality. This situation has led to the strategy of initiation “empirical” antifungal therapy prior to the detection of fungi. Meanwhile, improvements in diagnostic procedures are achieved, especially with imaging techniques and non-culture based methods which include antigen-based assays, metabolite detection and molecular detection of fungal DNA from body fluid samples using conserved or specific genome sequences. The AGIHO presents recommendations for the diagnosis of invasive fungal infections with risk-adapted screening concepts for the neutropenic

and febrile episodes of patients with hemato-oncological disorders.

Keywords Fungal infections · Diagnosis · Neutropenia · Cancer

Categories of evidence used in this guideline [20]

Category, Grade	Definition
Strength of recommendation	
A	Good evidence to support a recommendation for use
B	Moderate evidence to support a recommendation for use
C	Poor evidence to support a recommendation
D	Moderate evidence to support a recommendation against use
E	Good evidence to support a recommendation against use
Quality of evidence	
I	Evidence from ≥ 1 properly randomized, controlled trial
II	Evidence from ≥ 1 well-designed clinical trial, without randomization; from cohort or case-controlled analytic studies (preferably from >1 center); from multiple time-series; or from dramatic results from uncontrolled experiments
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees

Introduction

Invasive fungal infections are an increasingly important cause of morbidity and mortality particularly in patients with hematological malignancies [3, 11]. The most common fungi causing invasive infections in this setting are *Aspergillus spp.* and *Candida albicans*, but *non-albicans Candida* and a growing number of other organisms (e.g. *Mucor*, *Trichosporon*) are found with increasing frequency. The most important risk factors are prolonged severe neutropenia (neutrophil count of $<500/\mu\text{L}$ >10 days), allogeneic bone marrow / peripheral blood stem cell transplantation and subsequent intensive chemotherapy regimens after previous invasive fungal infections. The definite diagnosis of invasive fungal infections is difficult [6]. Although an early, empirical antifungal therapy in neutropenic patients with pulmonary infiltrates or refractory fever is mandatory in high risk patients [21], diagnostic efforts should be intensified. The identification of fungi are important for the choice of antifungals and duration of therapy, surgical intervention, monitoring of fungal manifestations or secondary antifungal prophylaxis.

Definitions

Because of substantial controversy concerning optimal diagnostic criteria for invasive fungal infections (IFI), the Invasive Fungal Infections Cooperative Group (IFICG) of the European Organisation for Research and Treatment of Cancer (EORTC) and the US Mycoses Study Group (MSG) have recently published an international consensus for patients with cancer and hematopoietic stem cell transplantation. The definition comprises three elements such as host factors, major or minor clinical criteria and microbiological criteria which could help to assign a degree of probability to the diagnosis. Three levels of probability are proposed: proven, probable and possible IFI [1]. These definitions are now widely used in Germany for clinical studies but much less for “bed-side” decision making.

Diagnostic procedures

For definite diagnosis of proven IFIs histological and cultural evidence from tissue biopsies or resection material or positive cultures from normally sterile body fluids is required (A III). However it is not always possible to fulfill these criteria in an early stage of infection. Moreover, the decision to start antifungal therapy in the neutropenic host is mostly based on the degree of neutropenia, presence of fever refractory to antibiotics or pulmonary symptoms with detection of pulmonary infiltrates on a thoracic CT scan. Current methods for diagnosis of systemic fungal infections include signs and symptoms, microscopy and culture techniques, serology, imaging procedures, endoscopic methods and biopsies. In addition to conventional mycological tests most promising are the detection of *Aspergillus galactomannan* (*Platelia Aspergillus Elisa*) and the polymerase chain reaction (PCR) based detection of fungal DNA in blood or bronchoalveolar lavage fluid. However, the PCR is intensively studied in Germany, but still remains an “in house” method in selected centers and a commercial test is not available.

1. Signs and symptoms

Local infections such as oral candidosis may not always be distinguished visually from mucositis secondary after chemotherapy. Furthermore, symptoms of IFI are non-specific, particularly in early stages. Bloodstream infections do not differ clinically in bacterial and fungal infections. Unexplained fever despite broad spectrum antibiotics for more than 3 to 6 days or recurring febrile episodes after initial defervescence and/or presence of pulmonary infiltrates during antibiotic treatment may indicate a fungal infection. Pulmonary aspergillosis may initially cause pleural pain, during neutrophil recovery hemoptyses may occur. Many hematologists start empiric antifungal therapy in a leukemic patient with pleural pain

and pulmonary infiltrate but other non-fungal infections need to be excluded as well (C III). Sinusitis may be an indication of a mould infection. Fungal esophagitis is mostly caused by *Candida* species primarily *Candida albicans*. However, similar to oral candidosis severe mucositis may mimic dysphagia and retrosternal burning which frequently occur after high-dose cytosine arabinoside and the diagnosis of esophageal candidosis can not be definitely established on clinical symptoms (C III). Skin infiltration during pancytopenia may be misinterpreted as thrombocytopenic purpura and can be caused by both yeasts and moulds. The clinical signs of hepato-splenic candidiasis (persistent fever, hepato-splenomegaly, increase of alkaline phosphatase) as well as of fungal endophthalmitis (uveitis posterior with white vitreous body infiltrates) typically develops during or after neutrophil recovery. In systemic candidosis, other organs (e.g. heart, kidney, bone) might as well be affected. In patients with hematological malignancies, mycoses of central nervous system (intracerebral lesions) are usually caused by moulds (*Aspergillus* species), rarely meningo-encephalitis caused by *Cryptococcus neoformans* may occur. All neutropenic patients with new neurologic symptoms such as seizure, changes in mental status or persistent headache should have a CT, or when available a MRI scan of the brain (A III). Cerebral aspergillosis should be regarded as the most likely infectious complication in the brain in patients with prolonged and severe neutropenia (B III).

2. Microscopy

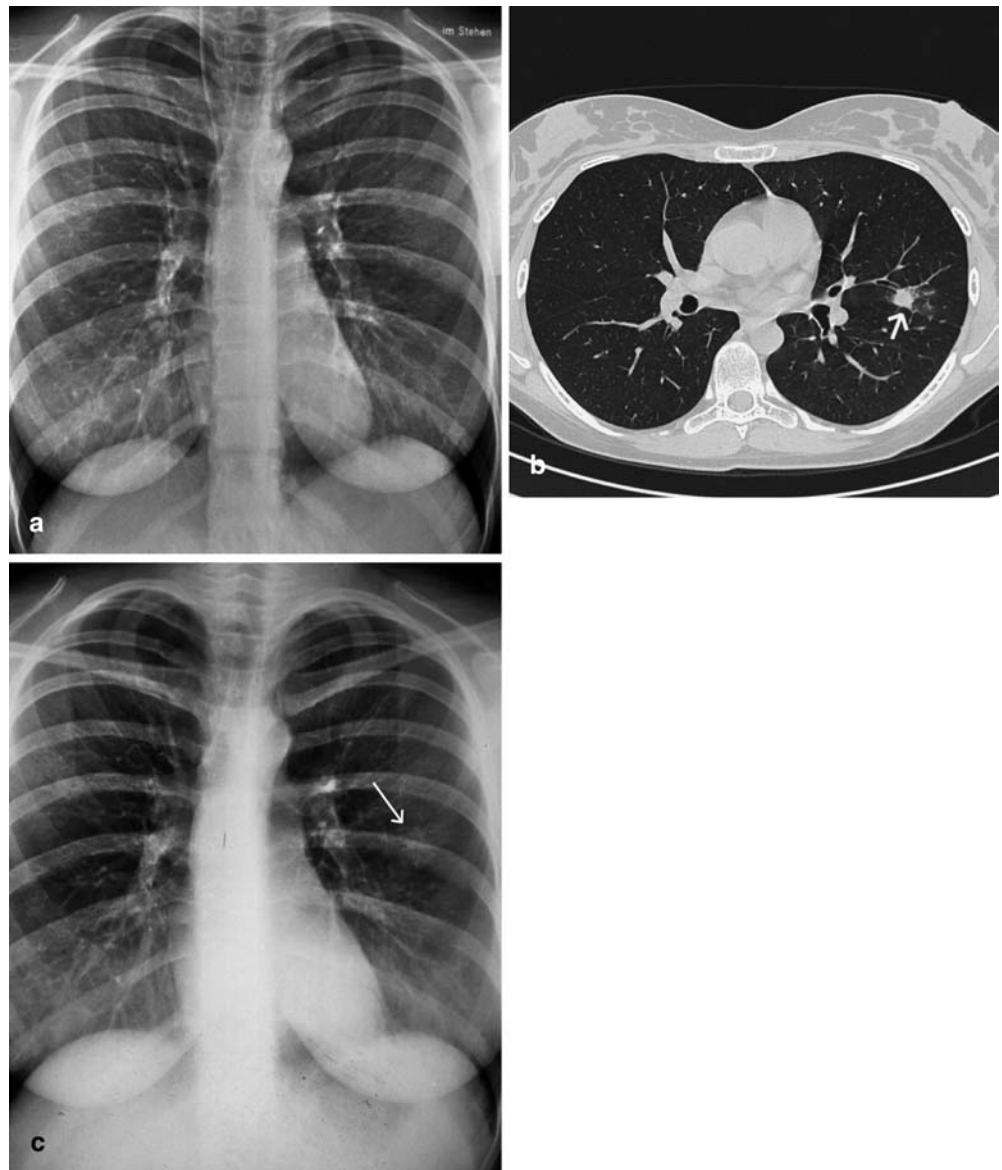
All tissues from patients with suspected IFI should be stained with fungal stains [7]. In direct preparations, Gram's staining or hematoxylin-eosin staining, fungi can easily be missed or misinterpreted as artifacts, fibrin filaments or necrotic fibers (B III). Hyphae are best visualized by "special fungal" stains and bronchoscopic material or tissue biopsies have to be examined with periodic acid Schiff reaction (PAS) or silver-methenamine technique (Grocott) (A III). Necrotic fungal structures become better visible with Grocott staining than with the PAS reaction, but at least one of these two staining procedures should be performed. Thrombosed vessels in biopsy or autopsy material should always be examined very carefully by the pathologist and numerous cutting sections should be analyzed. Evidence by fluorescent microscopy of fungi following the use of optical brightener such as calcofluor white treatment (possibly in combination with KOH) is an important addition to the methods listed above and may allow rapid diagnosis (B II). Cerebrospinal fluid (CSF) fluid should be cultured and antigen tested for *Cryptococcus neoformans*. *Cryptococcus neoformans* in CSF is detected by direct ink preparation in addition to antigen test by agglutination assay (A III). Microscopic differentiation between yeasts and hyphomycetes is easily achieved. By means of direct microscopic examination of hyphal fungi, important

parameter, like septal formation, diameter or ramification of the hyphae can be obtained. Microscopic differentiation of *Aspergillus spp.* and *Mucoraceae* may be improved by immunohistochemical examination. However, it is not always possible to clearly identify the species.

3. Culturing techniques

Reliable results can be obtained from normally sterile body fluids (e.g. blood, pleural effusion, CSF) and biopsy material. All fungi obtained from sterile sites should be identified to the species level (A II). Despite the fact that about 50% of patients with autopsy-proven invasive candidosis did not have positive blood cultures at lifetime, obtaining multiple blood cultures are the method of choice to detect fungemia (A III). In blood cultures, mainly yeast fungi can be isolated. To ensure the best level of detection at least two aerobic blood culture bottles with each 10 ml blood should be cultivated, instead of using one aerobic and one anaerobic blood culture bottle (A II). The methods used today (e.g. Bactec Mycosis-IC/F Medium) are believed to detect *Candida spp.* in up to 60% of all fungemias within 2–5 days [28]. Special methods such as lysis centrifugation (Dupont Isolator) may even improve the detection of fungi, but could lead to false positive results in neutropenic patients (B II). Lysis centrifugation is not regarded as standard method to cultivate fungi from blood cultures in neutropenic patients [25]. Evidence of moulds in sputum must be regarded to be a possible indicator of fungal pneumonia, yeasts however belong to the physiological flora of the gastrointestinal tract and may be regarded as contamination until invasive disease is proven by lung biopsy. All *Candida* isolates need to be identified to the species level and not only differentiated between *C. albicans* and *non-albicans Candida* (A III). Various selective differentiation agar (e.g. CHROMagar) are now available and could help a faster identification of *Candida* species according to the different colour on the agar plate. A funguria in a symptomatic patient without urinary catheter may be interpreted as an indication of a systemic fungal infection [6]. Materials should be cultured on special media enriched by antibiotics, preferably at 26°C and 37°C over a prolonged period of time (at least 7 days). The use of enriched media, e.g. Sabouraud bouillon, may be helpful for the isolation of fungi. The reference procedure for susceptibility testing of antifungal agents against yeasts and moulds is described by the National Committee of Clinical Laboratory Standards (NCCLS). Comparable standards for yeasts in Germany have been established by the DIN Norm (DIN 58940–84), but the medium (high resolution medium) used in this test differs from the RPMI 1640 medium in the NCCLS standard [8, 10, 26]. In routine practice, most mycology laboratories use the E-Test as a suitable alternative method due to its easy handling and good reproducibility [16].

Fig. 1. a–c 23-year-old female suffering from acute lymphoblastic leukemia and fever. Despite of unsuspecting initial chest x-ray (**a**), HRCT demonstrated ill-defined nodules with halo-sign in left upper lobe (**b**, →). These findings became visible during follow-up on chest x-ray as well (**c**, →)



4. Serology

Several antibody and antigen test methods have been applied for the diagnosis of invasive candidosis or aspergillosis. The antibody tests have sensitivities in the range of 17–90% and may be only useful in the combination with antigen tests. Furthermore, currently available antibody tests may show a delayed or decreased reaction in immunosuppressed patients [7]. Antigen testing is established for *Candida* and *Aspergillus* species, *Histoplasma* and *Cryptococcus neoformans*. In immunocompromised patients, the cryptococcal CSF antigen is positive in many cases of cryptococcal meningitis. However, most reports focused on diagnosing cryptococcal meningitis in AIDS patients and not patients with malignancies. For the antigen testing of *Candida* the “Cand-Tec®” is frequently used, a latex agglutination test with an antigen, not specifically defined. A sensitivity of

up to 70% (range 30–70%) and a specificity of 88% have been reported [24]. An ELISA testing format for detecting *Candida mannan* antigen (Platelia® *Candida*) that has been introduced may be the most sensitive method, but is still under investigation. Routine testing for *Candida* antibodies or antigen is not recommended for patients with hemato-oncological disorders (C III).

Aspergillus antibodies are frequently not detectable in immunocompromised patients. However, testing for antigens has produced promising results. For the galactomannan assay (Pastorex® *Aspergillus*) a specificity of 90–100% and sensitivity of 26–76% has been documented [12, 22]. More recently, good results have been achieved with the ELISA testing format for galactomannan (Platelia® *Aspergillus*). Based on several studies and two large clinical series in neutropenic patients the test showed a sensitivity of 80–100% and a specificity of >90% with a positive predictive value of 87.5% [23, 32].



Fig. 2a, b Hepatosplenic candidosis. **a** Wheel-in-wheel structures—a characteristic sign of an early stage of candidiasis in the liver— at a size of 5 mm shown in the picture on the left. On the right-hand side of the picture, microabscesses close to vessels can be seen. **b** Spleen with hypoechoic lesions from *Candida spp.*

Galactomannan in serum is transient and it is recommended that twice weekly testing is performed (A II). Another new method for serological diagnosis which is being tested is the so-called G-Test for the detection of 1–3-beta-D-Glucans (Fungitec®G) [27]. However, data on sensitivity and specificity are limited.

Routine testing for *Aspergillus* antibodies is not recommended for patients with hemato-oncological disorders, but antigen detection with the ELISA galactomannan test is useful in this patient population.

5. Imaging procedures

Early stages of pulmonary aspergillosis are often inconspicuous in conventional chest x-ray (Fig. 1) [14]. At high resolution computed tomography (HRCT) or thin-section multislice CT however, typical infiltration patterns can be detected at an early stage (Fig. 1) [14] (A II). They consist of small nodules with an halo sign and localized in the vicinity of the vessels [5]. The specificity of the halo sign is limited, since several differential diagnoses such as bleeding, embolism, leukemic infiltrates have to be considered as well beside of the most frequent underlying fungal infection [17, 19] (B II). Cavitations and the air-crescent sign typically occur after hematopoietic reconstitution [19] (B III). In the course of candidemia, liver and spleen are frequently involved (B III). A central hyperechoic lesion with a hypoechoic rim (size 5–20 mm) can be detected by ultrasound [18] (Fig. 2) (B II). An early diagnosis of organ infiltration however, can also be made by computed tomography or magnetic resonance tomography (MRI), but MRI seems to be the more sensitive method [30] (B III). In case of neurological symptoms, a CT scan of the cranium is indicated in case

of emergencies (B III), otherwise a MRI should be preferred, because MR is the method of choice for the examination of the cerebral parenchyma and the meninges [2] (B III). Nevertheless, for diagnosis of the paranasal sinuses computed tomography is more reliable than MRI for assessing the bone structure (B III). Conventional imaging of the paranasal sinuses should be avoided due to the necessity to image the ethmoidal and sphenoidal sinuses [29] (D III). Fungal retinitis or endocarditis are very rare in neutropenic patients, but ophthalmoscopy and echocardiography should be performed if candidemia has been diagnosed.

6. Endoscopic methods

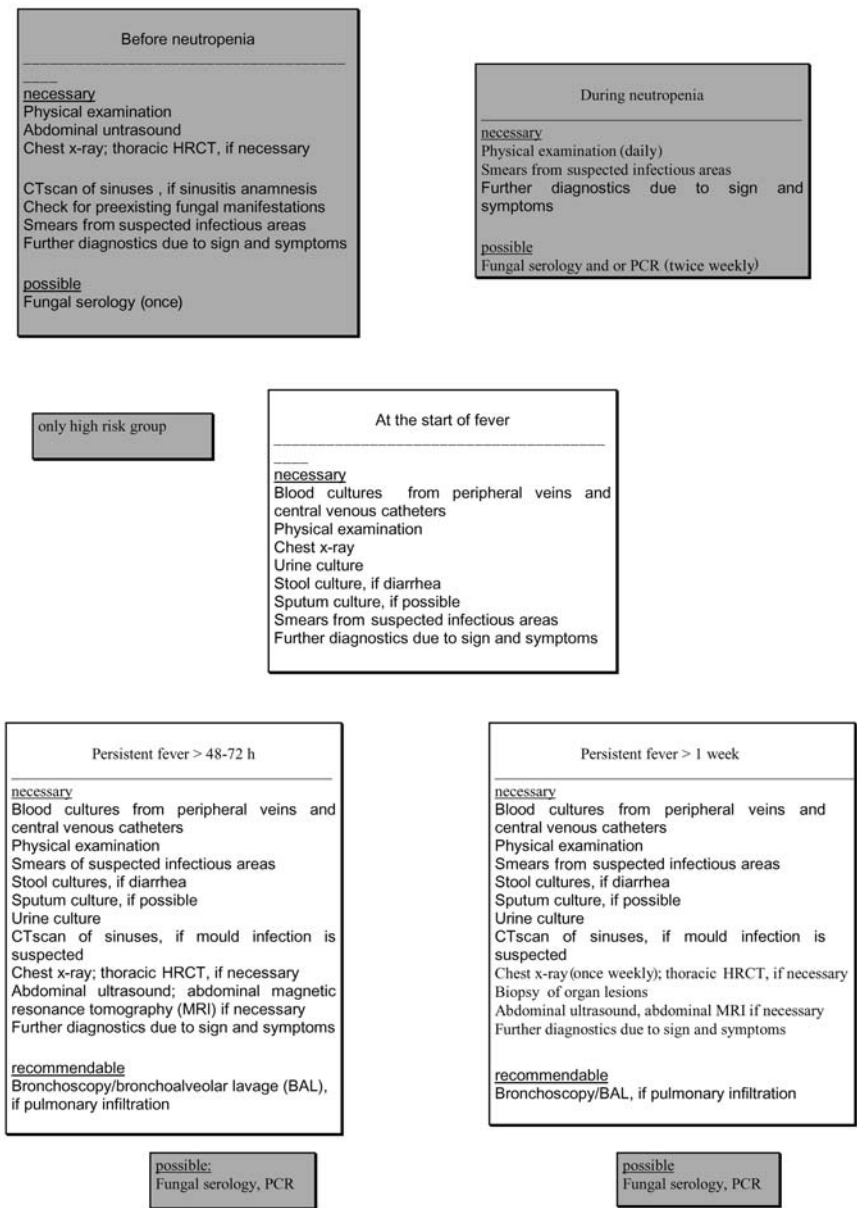
In the presence of pulmonary infiltrates, especially in diffuse processes, bronchoscopy with bronchoalveolar lavage may be useful [15]. However, the value of BAL is not properly studied and the sensitivity appears to be about 50% (B III). Procedures and review of the literature for pneumonia, other documented infections, septicemia and fever of unknown origin are described in detail in the respective papers in this issue. In case of therapy-resistant esophagitis or gastric pain, esophago-gastro-duodenoscopy (preferably with biopsy) should be performed (A III).

7. Organ biopsy

If clinically possible, biopsies of suspected areas (skin, pulmonary, hepatic lesions) should be obtained (A II). Transcutaneous needle biopsies of peripheral pulmonary foci have a diagnostic yield of 50–80% and the rate of complications is low. Liver biopsies, on the contrary, show pathogenic evidence less frequently. If possible, an autopsy should be performed on deceased patients to collect important epidemiologic information (A II).

8. Polymerase chain reaction (PCR)

Since the early 1990's, several methods for detecting fungi-specific DNA by means of hybridization and amplification of nucleic acids have been developed. Two approaches have been studied so far, first, to look for genus-specific genomic sequences as well as single copy genes or, second, to look for multiple copy genes which could be detected in almost all fungal species. Primers from either the 18ssu-rRNA subunit gene, the 28S rRNA gene or mitochondrial genes have been studied as a so-called "Panfungus PCR". The Panfungus PCR is followed by hybridization with species-specific probes to gain adequate specificity. The Panfungus PCR using a sequence from the 18ssu-rRNA gene followed by species-specific hybridization with probes for *Candida* species as well as *Aspergillus* species has been studied most extensively in several clinical studies in hemato-oncological patients [9, 13]. The specificity is around 65–75%

Fig. 3 Diagnostic procedures: overview

depending on the number of tests needed to establish the diagnosis with a sensitivity of 100%. The species-specific approach using a nested PCR (two-step PCR) with primers derived from a variable region of the 18S rRNA gene which is highly conserved among *Aspergillus* species have been studied in BAL and blood [4, 31]. The test specificity was 89%. The molecular diagnostic approach is very promising with high sensitivity and specificity (B II), however, fungal PCR is not yet a standardized nor widely available procedure.

Diagnostic schedules

1. Screening before manifestation of infection

The aim of diagnostic screening is the localization of possibly pre-existing fungal manifestations and information about the type of colonizing agents. The predictive value of "surveillance cultures" (e.g. pharyngeal and mouth washings, sputum, stool, nasal swabs) is controversially discussed. Depending on the individual experience of the centers the method might be used. Initial abdominal ultrasound and chest x-ray are recommendable. Serological tests are optional. Regular screening programs (see Fig. 3) are only necessary for patients with a high risk for invasive fungal infections.

2. Diagnostic procedures in febrile neutropenia

If fever occurs, the diagnostic routine procedure listed in Fig. 3 is recommended. Antimicrobial treatment should begin immediately after collection of blood cultures and must not be delayed by further diagnostic measures [2]. If defervescence cannot be achieved within 72 to 96 hours of antibacterial treatment, the diagnostic procedure has to be repeated regularly, also after initiation of antifungal treatment. The detection of other rare pathogens or a delayed identification of an invasive fungal infection is important. If *Candida spp.* have been isolated from blood cultures or from central venous catheters, the following examinations are recommended: a) serial blood cultures during fever, and two to three times before and after antifungal treatment, b) ophthalmoscopy and abdominal ultrasound (plus MRI if necessary) initially and after neutrophil recovery, and c) echocardiography (optionally).

In summary, a combination of various methods with regular screenings is necessary for early diagnosis of invasive fungal infections.

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