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Improved detection of medically important fungi by immunoperoxidase staining with polyclonal antibodies

Abstract This study was performed to identify pathological fungi of eight species [Aspergillus fumigatus, Candida albicans, Torulopsis (Candida) glabrata, Cryptococcus neoformans, Fusarium anthophilum, Rhizopus oryzae, Sporothrix schenckii and Trichosporon beigelii] in formalin-fixed, paraffin-embedded tissue sections by indirect immunoperoxidase staining. Mature albino rabbits were immunized with formalin-killed organisms. Antibodies were prepared by precipitation. Immunoperoxidase staining was applied to the paraffin-embedded tissue sections of experimentally infected mice and human autopsy and surgical specimens. Although the cell walls of each fungus stained clearly, many cross-reactivities appeared. However, it was possible to obtain specificity for the eight species by absorption and dilution of the antisera.

Key words Indirect immunoperoxidase staining • Tissue section • Fungi • Immunohistochemistry • Polyclonal antibody

Introduction

In the past three decades, the incidence of deep-seated fungal infections has increased markedly following the long-term use of broad-spectrum antibiotics, steroids, immunosuppressive and cytotoxic agents. The histopathological diagnosis of mycoses usually depends on the morphological appearance of the lesions combined with cultural identification. For clinical laboratories, the detection of pathogenic fungi is necessary as a prelude to therapy. The identification of specific fungal infections is sometimes difficult in formalin-fixed, paraffin-embedded tissue sections from autopsy and surgical specimens as all have similar morphologies. Several histochemical procedures are useful for the detection of fungal elements in tissue sections, such as the periodic acid-Schiff reaction (PAS), Gomori’s methenamine-silver procedure (Grocott) and mucicarmine stain and these enable us to determine the infected strain in specimens containing many fungal elements. However, it is not easy to ascertain the pathogenic strain in cases with few mycelia or other structures in biopsy specimens. Fluorescent antibody (FA) techniques have been used as one of the most reliable methods [5, 7, 10], but in recent years, immunoperoxidase staining has been commonly used for tissue sections. Since various techniques have been developed including the direct and indirect peroxidase techniques [19, 20] and avidin-biotin-peroxidase methods, they had been used to detect fungi [13, 17, 21]. The problem in such application is that the commercially available antisera to fungi, even though they can be applied for serological diagnosis, have unavoidable cross-reactions among some species [22]. In view of this limitation, we tried the indirect immunoperoxidase method to identify four species including Aspergillus (A.) fumigatus, Candida (C.) albicans, Cryptococcus (Cr.) neoformans, and Fusarium (F.) anthophilum, which are commonly encountered in routine practice and sometimes difficult to distinguish from one another with haematoxylin and eosin (H&E), PAS and Grocott’s procedures (see [15]). The growing number of patients with acquired immunodeficiency syndrome (AIDS) may contribute to the recent increase in opportunistic fungal infections [1]. In response to an increasing demand for detection of pathogenic fungi, we applied the methods of our previous study and attempted to differentiate among the eight medically important fungi immunohistochecmically with polyclonal antisera specific to each species. These included four new species: Torulopsis (T. or C.) glabrata, Rhizopus (R.) oryzae, Sporothrix (S.) schenckii and Trichosporon (Tr.) beigelii.

Materials and methods

Preparation of immunogens

The immunogens were prepared from A. fumigatus strain IAM-2007 [14], C. albicans MCLS-2 [18], T. (C.) glabrata 1312, Cr.
neoforans CDC 551, F. anthophilum [4, 8], R. oryzae IFO 4783, S. schenckii TIMM 0960 and Tr. beigelii TIMM 1526. Each strain was cultured in the appropriate conditions: Sabouraud's dextrose agar for C. albicans, Cr. neoformans, T. (C.) glabrata, and F. anthophilum; potato dextrose broth or agar for A. fumigatus, R. oryzae and Tr. beigelii, and brain heart infusion agar for S. schenckii. Each strain was cultured at 25°C for 6 days. Whole cells were killed by 1% formalin for 24 h, washed three times in sterile physiological saline and finally suspended in sterile physiological saline to give a concentration of 5x10^7 cells/ml. The conidia of A. fumigatus and microconidia of F. anthophilum were filtered through sterilized and piled gauze to remove hyphae, before the final adjustment.

Preparation of antisera

Volumes of 0.5, 1, 2 and 4 ml of each suspension were injected intravenously into four mature albino rabbits for each strain weighing about 3.0 kg, at 4 day intervals. Crude polysaccharide derived from each fungus was prepared by the method as described elsewhere [25] and used as an antigen for precipitation assay. At 3 weeks after the first injection, if titres of 1:16 or more were developed by precipitin test against crude polysaccharide (100 μg/dl), whole antiserum was harvested from each immunized rabbit and the antiserum was used for further experiment. When these antisesra had cross-reactivities to other strains, they were absorbed by corresponding strains in order to obtain species-specific antibodies.

The following is an example of a cross-reactivity resolution.

The antiserum to T. (C.) glabrata was absorbed by incubating the serum with formalin-killed cells of C. albicans at 37°C for 2 h, then at 4°C for 12 h (serum: formalin killed-cell=2 ml:1 g wet weight). The supernatant was collected by centrifugation (15000 rpm, 30 min). We controlled these procedures by the results of staining tests.

Preparation of tissue specimens

Eight specific pathogen-free, 4-week-old male, ICR mice were used for each strain. Cyclophosphamide (200 mg/kg) and prednisolone (70 mg/kg) were injected intraperitoneally into each mouse to suppress immune systems and host defence reactions. The fungi were cultivated on agar slants for 4 days at 25°C as stated above. The viable spore, conidia and microconidia of each organism were filtered through sterilized and piled gauze to remove hyphae, before the final adjustment, immediately fixed in phosphate-buffered 3% paraformaldehyde, processed routinely and finally embedded in paraffin. If the mice did not have sufficient fungal lesions to use in this experiment, we tried the same protocol again. The care and use of the animals reported on in this study were approved by the Animal Care of Shinsu University School of Medicine. In addition, formalin-fixed, paraffin-embedded sections obtained from three human autopsy cases and one surgical case (one of aspergillosis, two of candidiasis, one of zygomycosis) from our department were examined. Ethical approval was sought and informed consent obtained in all human cases.

Procedure for staining

Sections were available for H&E, PAS and Grocott. The indirect immunoperoxidase method was used as described previously [15]. Briefly, the endogenous peroxidase activity of these tissue sections was eliminated by absolute methyl alcohol containing 0.3% hydrogen peroxide for 30 min. Deparaffinized sequential 4 μm sections were then incubated for 1 h with appropriately diluted antisera as primary antibodies, described previously. After washing three times with phosphate buffer saline (PBS), the horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin (1:50 dilution, DAKO, Japan) was applied for 1 h as a secondary antibody. After washing with PBS, the peroxidase reaction was accomplished for about 5 min with TRIS buffer containing 0.2 mg/ml 3,3'-diaminobenzidine, 0.005% hydrogen peroxide and 0.20 mg/ml sodium azide. Sections were then counterstained with methyl green, dehydrated and mounted for examination by light microscopy. Control sections were stained by simply omitting the primary antibody or with the primary antibody substituted by nonimmunized rabbit serum, all of which gave negative immunoreactivities for any fungal element and no significant background staining.

Results

The precipitin titres of each antiserum are summarized in Table 1. Although the antibodies showed many cross-reactivities among several species, the application of a simple absorption technique, as stated above, completely abolished these cross reactions, yielding highly specific and sensitive antibodies that satisfy further use for immunohistochemical staining.

The staining properties of the fungi before absorptions are listed in Table 2. These antibodies showed many cross-reactions between the strains. Staining properties following absorption, and the strains used in absorption are shown in Table 3.

Table 1 Precipitin titres against crude polysaccharide (100 μg/ml) of each organism [Af Aspergillus fumigatus, Ca Candida albicans, Cn Cryptococcus neoformans, Fa Fusarium anthophilum, Ro Rhizopus oryzae, Ss Sporothrix schenckii, Tg Torulopsis (C.) glabrata, Tb Trichosporon beigelii]

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