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

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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 preclude 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 immunohistochemically 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.*

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neoformans 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 5×10^7 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 and 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 antisera 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 were separately suspended in sterile saline at a concentration of 1×10^8 cells/ml. A volume of 0.25 ml of this suspension was then injected via the tail vein at 48 h after administration of immunosuppressive agents except for *S. schenckii*. Suspension of *S. schenckii* was injected intraperitoneally. The same doses of immunosuppressive agents were injected 2 days after the injection of the fungal suspension. At 4 days after the second immunosuppression treatment, three of these mice were sacrificed by cervical dislocation, 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 Shinshu 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. Staininig properties following absorption, and the strains used in ab-

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*]

Antibody	Antigen							
	Af	Ca	Cn	Fa	Ro	Ss	Tg	Tb
Af	1:16	–	–	–	–	1:16	–	–
Ca	1:2	1:64	–	–	1:2	1:2	1:32	–
Cn	–	1:2	1:64	–	–	–	–	1:2
Fa	–	1:2	–	1:64	–	–	1:2	–
Ro	1:2	–	–	–	1:16	–	–	1:1
Ss	1:8	–	–	–	1:8	1:16	–	–
Tg	1:2	1:8	–	–	–	1:2	1:16	1:4
Tb	1:4	1:1	1:16	–	–	1:1	1:8	1:64

Table 2 Staining titres of each antibody by indirect immunoperoxidase staining before absorption

Antibody	Antigen							
	Af	Ca	Cn	Fa	Ro	Ss	Tg	Tb
Af	1:3200<	1:200	1:800	1:800	1:400	1:1600	1:800	1:400
Ca	1:3200	1:3200<	–	1:400	1:400	1:1600	1:1600	–
Cn	1:400	1:3200	1:3200<	1:800	1:800	1:800	1:1600	1:3200
Fa	1:800	1:3200	1:400	1:3200	1:800	1:800	1:1600	1:800
Ro	1:3200	1:400	1:400	1:800	1:3200	1:1600	1:1600	1:800
Ss	1:1600	1:400	–	1:3200	1:400	1:3200<	–	1:400
Tg	1:400	1:3200	–	–	1:400	1:1600	1:3200	1:800
Tb	1:800	1:400	1:1600	1:1600	–	1:200	–	1:3200<

Table 3 Staining titres of each antibody by indirect immunoperoxidase staining after absorption (maximum dilution of antiserum showing positive reaction)

Antibody	Antigen								
	Af	Ca	Cn	Fa	Ro	Ss	Tg	Tb	Absorption*
Af	×3200	–	–	–	–	–	–	–	Ca
Ca	–	×3200	–	–	–	–	–	–	Af
Cn	–	–	×3200	–	–	–	–	–	Ca
Fa	–	–	–	×1600	–	–	–	–	Ca
Ro	–	–	–	–	×3200	–	–	–	Af
Ss	–	–	–	–	–	×1600	–	–	Fa
Tg	–	–	–	–	–	–	×3200	–	Ca
Tb	–	–	–	–	–	–	–	×1600	Fa

* Strain used in absorption

sorption, are listed in Table 3. Cross-reactivities after absorption were observed in dilutions of up to 1:1600, but disappeared over 1:1600. The optimal dilutions for *A. fumigatus*, *C. albicans*, *T. (C.) glabrata*, *Cr. neoformans* and *R. oryzae* were 1:3200, and those for *F. anthophilum*, *S. schenckii* and *Tr. beigelii* were 1:1600. Fungal elements were clearly stained with specificity in such dilutions after absorption by the indirect immunoperoxidase method (Figs. 1–8). Generally in this series when comparing intensity of positive reaction of antisera before and after absorption it was clear that positive reactions were weaker when using of absorbed antisera than non-absorbed ones.

In order to validate the practical use of our antibodies for diagnosis of human mycoses, we selected several cases of fungal infections as shown in Table 4, in which mixed infections were suspected but the causative organism(s) was not identified by routine histochemical procedures. The indirect immunoperoxidase staining with appropriate dilutions of the antibodies revealed the fungal elements, unequivocally demonstrating that all the cases were caused by a single species.

Discussion

Some investigators have reported that immunohistochemical examination is valuable in the detection of fungi using polyclonal [12, 13, 15, 17, 22], monoclonal [21] and commercial antibodies. Preparations of monoclonal antibody are expensive and time ensuring but commercial antibodies have much cross-reactivity [6, 22]. In the view of these limitations we examined polyclonal antibodies for immunohistochemistry. The results of this study indicate that immunoperoxidase staining is useful for the diagnosis of fungal infections even in formalin-fixed and paraffin-embedded specimens. By the positive colour intensities of the species and disappearance of cross-reactivities we established optimal dilution titers. There have been many reports concerning the cross-reactions among fungi within the genus [5, 12, 16, 23]. Hotchi stated that it was difficult to eliminate cross-reactivities among the species by absorption and dilution [5] and that commercial polyclonal antibodies have many cross-reactivities [6, 22]. Because the cell wall polysaccharides constitute the major antigenic determinants, some of which are shared by apparently different organisms (*Cr. neoformans* and *Tr. beigelii* share common antigenicity [16]. Rather than diverting the commercially available antibodies to immunohistochemical staining, highly specific and sensitive polyclonal antibodies can be prepared by means of the relatively simple and economic procedures. In our study, both absorption and dilution allowed the distinction of the eight different fungi from each other. However, even after most cross-reactivities were eliminated by absorption and dilution of the antisera, there was still a limit in the application of this procedure since strong cross-reactivity cannot be completely abol-

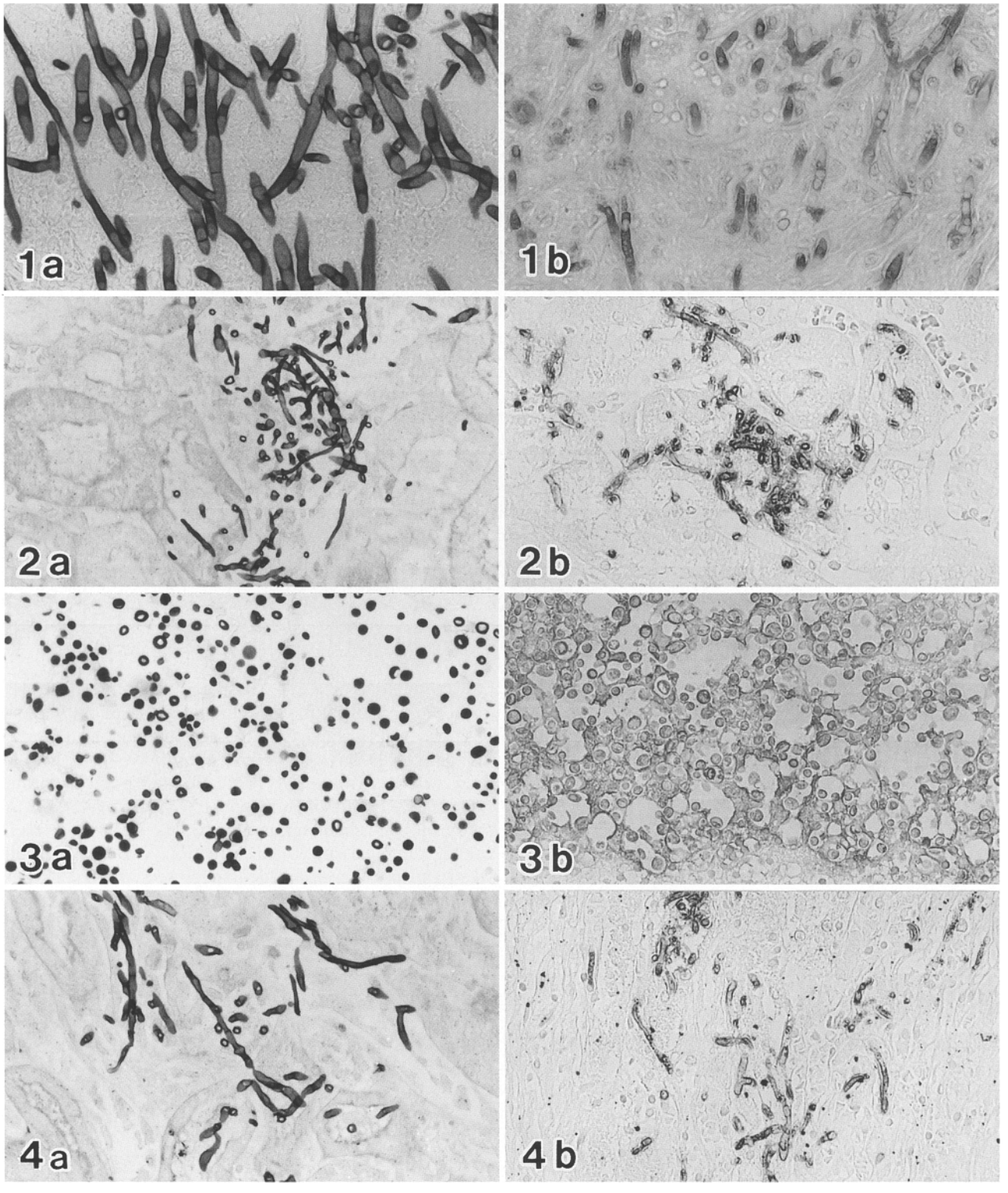


Fig. 1a *Aspergillus* (*A.*) *fumigatus* in the lung of an infected mouse showing dichotomous branching and septation of hyphae. Grocott, ×100. **b** Positive immunoperoxidase stain of *A. fumigatus* in the same specimen as in **a**. ×100

Fig. 2a *Candida* (*C.*) *albicans* in the kindey of an infected mouse showing prominent hyphal and pseudohyphal strands with varying length and little formation of yeast. Grocott, ×100. **b** Positive immunoperoxidase stain of *C. albicans* in the same specimen as in **a**. ×100

Fig. 3a *Cryptococcus* (*Cr.*) *neoformans* in the brain of an infected mouse showing numerous yeast cells with relative uniformity in shape and size. Grocott, ×100. **b** Positive immunoperoxidase stain of *Cr. neoformans* in the same specimen as in **a**. ×100

Fig. 4a *Fusarium* (*F.*) *anthophilum* in the kidney of an infected mouse showing branching septate hyphae. The morphology is identical that of aspergillosis except for the difference in size. Grocott, ×100. **b** Positive immunoperoxidase stain of *F. anthophilum* in the same specimen as in **a**. ×100

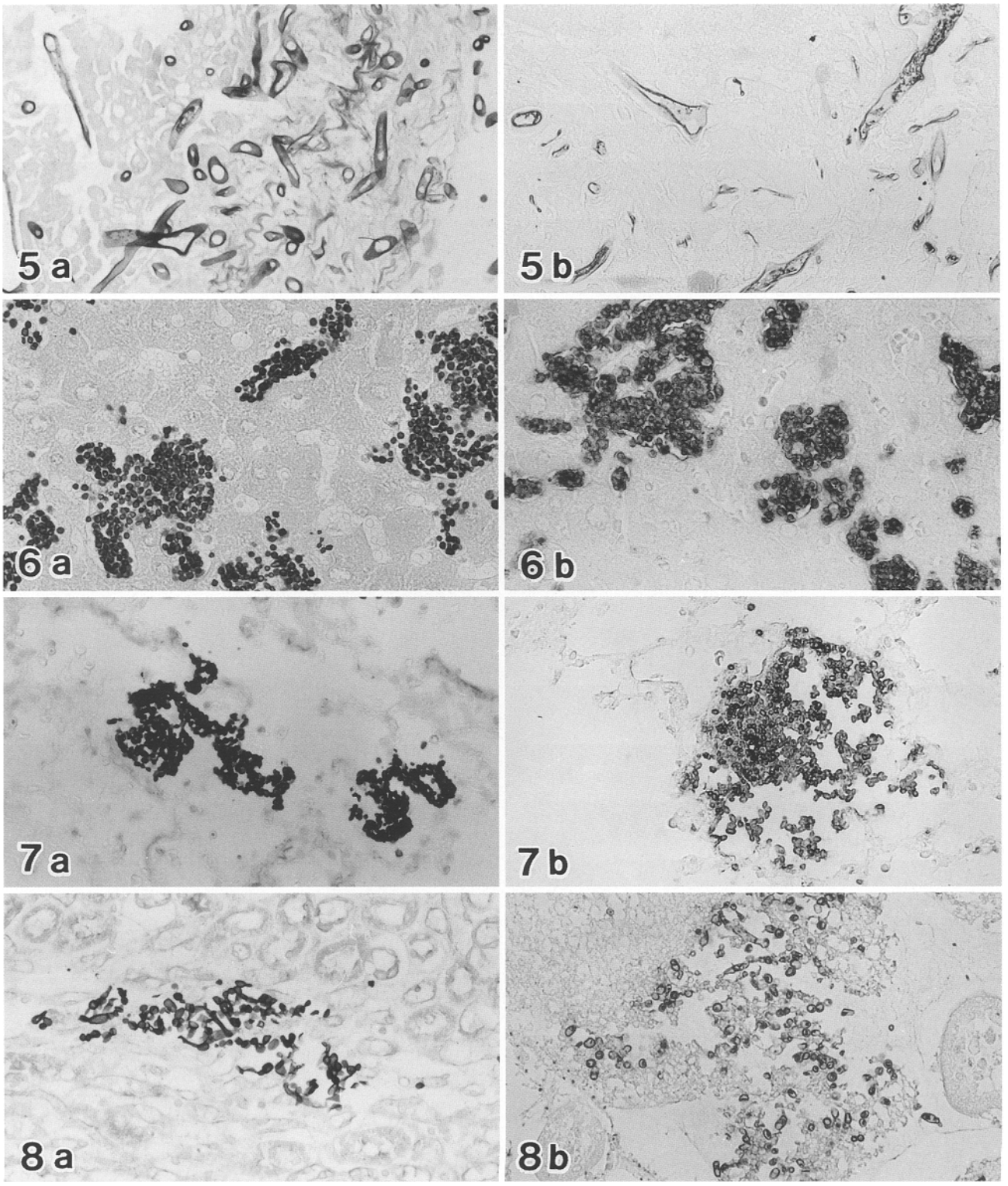
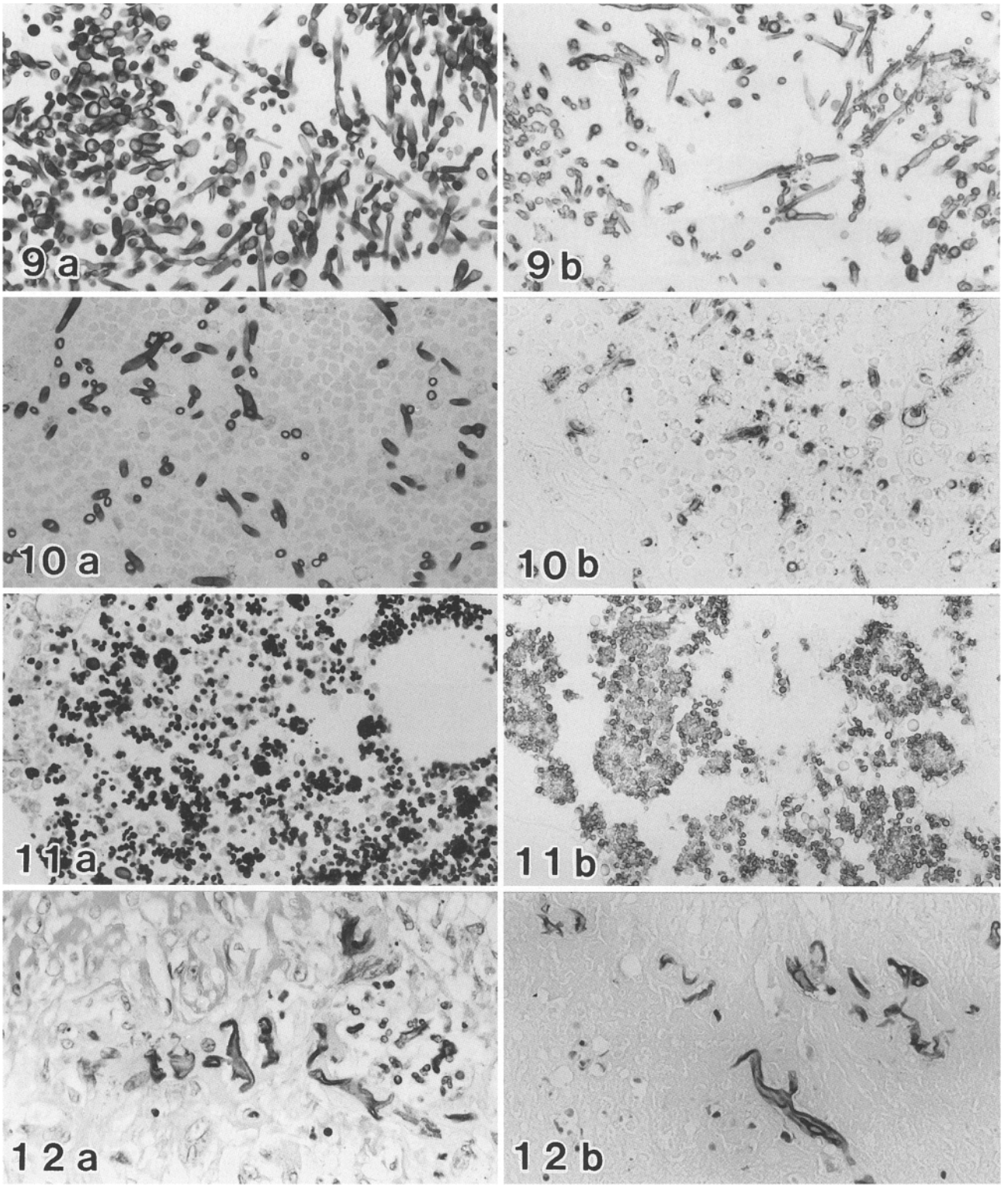


Fig. 5a *Rhizopus* (*R.*) *oryzae* in the liver of an infected mouse showing ribbon-like filamentous fungus with irregular broad, nonseptate hyphae. Grocott, ×100. **b** Positive immunoperoxidase stain of *R. oryzae* in the same specimen as in **a**. ×100

Fig. 6a *Sporothrix* (*S*) *schenckii* in the liver of an infected mouse showing massive conidial growth in sinusoids. Grocott, ×100. **b** Positive immunoperoxidase stain of *S. schenckii* in the same specimen as in **a**. ×100

Fig. 7a *Torulopsis* [*T.* (*C.*)] *glabrata* in the lung of an infected mouse showing nest of numerous round budding yeast cells without hyphal and pseudohyphal formation. Grocott, ×100. **b** Positive immunoperoxidase stain of *T.* (*C.*) *glabrata* in the same specimen as in **a**. ×100

Fig. 8a *Trichosporon* (*Tr.*) *beigelii* in the kidney of an infected mouse showing budding yeasts, arthroconidia and mycelium. Grocott, ×100. **b** Positive immunoperoxidase stain of *Tr. beigelii* in the same specimen as in **a**. ×100



ished for closely related fungal species. Determination of the absorption characterististics and further serotypic diagnosis is clinically and epidemiologically significant for some yeast-like fungi, such as *Torulopsis* (*Candida*) and *Cryptococcus* and these require cultural isolation and identification of the causative fungus, based on morphological features often aided by serological typing.

Recently, cases of mixed mycoses have been reported [2, 8, 9, 13], and it is readily assumed that the number of such cases will increase due to the prevalence of AIDS and the use of immunosuppressive therapy for transplantation. The specificity of these antibody will make a great contribution to differential diagnosis of mixed fungal infections showing morphological similarity in the same

Table 4 Profiles and histopathological diagnoses of human cases

Case	Clinical diagnosis	Age/Sex	Clinical outcome	Involved organ	Strain suspicious[a]	Immunohistochemical result[b]	Final diagnosis[c]
1	Graft versus host disease	73 Male	Died	Jejunum	Tg Tb Ca	Ca	Ca
2	Malignant lymphoma	66 Female	Died	Bronchus	Ca Af Fa	Af	Af
3	Malignant lymphoma	82 Female	Died	Renal pelvis	Ca Tg Cn Ss	Cg	Cg
4	Lung cancer	50 Male	Resolved	Lung	Af Ro	Ro	Ro

[a] Fungal structure revealed by haematoxylin and eosin, periodic acid-Schiff and Grocott suggested these strains. However, precise and final identification were not obtained by only these stainings (Figs. 9a, 10a, 11a and 12a)

[b] Using polyclonal antibody described as above (Figs. 9b, 10b, 11b and 12b)

[c] These results were decided by both fungal structure and immunohistochemical result

section, such as a small fungal focus composed of both *C. albicans* and *T. (C.) glabrata*. The practical use of these antibodies, therefore, may contribute to better immunohistological diagnosis of the aetiologic agents in formalin-fixed, paraffin sections. This experience may contribute to the diagnosis of fungal infections in autopsy cases and the fungal diagnosis in surgical and biopsy specimens.

The fungal strains used for immunization in this study, excluding some endemic pathogens such as *Histoplasma*, *Coccidioides* and *Paracoccidioides* which are rarely encountered in our country, cover the major mycotic pathogens responsible for opportunistic infections in immunocompromised patients. Several reports have shown antigenic differences among extracts of organisms in different growth phases [8, 11, 24]. Although our antigens were only spores and microconidia, all fungal elements had clearly positive staining by the indirect immunoperoxidase method. In addition, all the species had evidence of cross-reactivities in dilutions of up to 1:3200. It was frequently observed that after absorption by one strain, the serum lost its cross-reactivity not only to the strain used for absorption but to other strains. For example, anti-*C. albicans* serum had cross-reactivities to *T. (C.) glabrata*, 1:3200 and *F. anthophilum*, 1:1600. After absorption using *T. (C.) glabrata*, its cross-reactivity to *T. (C.) glabrata* disappeared. However, the absorption by *F. anthophilum* actually eliminated its cross-reactivities to both *T. (C.) glabrata* and *F. anthophilum*. This indicates that the same antigenicity among strains and fungal elments of one species, such as spores and hyphae, may exist. We think that fractions of monnoprotein, glycoprotein, and enzymes probably cause the antigenic differences among substrains or different growth phases [3, 26]. Therefore this procedure is not useful for the differentiation of fungal components including conidia, hyphae and pseudohyphae. In addition, in the same sections, varying staining capacity appeared along the cell walls which is an indication of a possible difference in the antigenic composition at different phases of fungal growth or dependence on the viability of the organisms. In comparison with the FA technique, the immunoperoxidase staining provides a permanent slide preparation available for an ordinary light microscope. In addition, this procedure produces more rapid diagnosis than culture and is useful for the detection of a small amount of fungal components in biopsy specimens, undetectable by PAS and Grocott's staining. The antibodies used in our study are species-specific and polyclonal. But as stated previously, in order to identify fungi in tissue sections by the indirect immunoperoxidase method, it is necessary to understand the antibody characteristics well. We conclude that indirect immunoperoxidase procedures using polyclonal antibodies are useful in confirming the diagnosis of fungal infections.

Fig. 9a *C. albicans* in the oesophagus of case 1 (autopsy case). This fungus was composed of yeasts and pseudohyphae. It is difficult to distinguish from *Tr. beigelii*, and mixed infection of *C. albicans* and *T. (C.) glabrata* was suspected. Grocott, ×100. **b** Positive reaction to the anti-*C. albicans* antibody in the same specimen as Fig. 9a. There was no reaction to anti-*T. (C.) glabrata* antibody and anti-*Tr. beigelii* antibody. ×100

Fig. 10a *A. fumigatus* in the bronchus of case 2 (autopsy case). This case had only a hyphal component. It is difficult to distinguish from *F. anthophilum* and *C. albicans*. Grocott, ×100. **b** Positive reaction to the anti-*A. fumigatus* antibody in the same specimen as Fig. 10a. There is no reaction to anti-*F. anthophilum* antibody and anti-*C. albicans* antibody. ×100

Fig. 11a *T. (C.) glabrata* in the renal pelvis of case 3 (autopsy case). This lesion was composed of nests of yeasts. It is difficult to distinguish from *Cr. neoformans*, *C. albicans* and *S. schenckii*. Grocott, ×100. **b** Positive reaction to the anti-*T. (C.) glabrata* antibody in the same specimen as Fig. 11a. There was no reaction to anti-*Cr. neoformans* antibody, anti-*C. albicans* antibody and anti-*S. schenckii* antibody. ×100

Fig. 12a *R. oryzae* in the lung of case 4 (surgical case). Cellular reaction includes giant cells, lymphocytic infiltration and granuloma formation. The fungal element in this case showed a bizarre shape. It was suspected that this fungus was *R. oryzae* or *A. fumigatus*. Grocott, ×100. **b** Positive reaction with the anti-*R. oryzae* antibody in the same specimen as Fig. 12a. There was no reaction to anti-*A. fumigatus* antibody. ×100

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