Original Article

Histopathological Evaluation of the Efficacy of Antifungals for Experimental *Trichosporon* Bloodstream Infection

Daisuke Sasai¹, Yoichiro Okubo^{1*}, Takao Ishiwatari¹, Takashi Sugita², Takehiko Kaneko³, Somay Yamagata Murayama⁴, Tsuyoshi Shimamura^{1,5}, Minoru Shinozaki¹, Chikako Hasegawa¹, Aki Mitsuda¹, Naobumi Tochigi¹, Megumi Wakayama¹, Tetsuo Nemoto¹, and Kazutoshi Shibuya¹

¹Department of Surgical Pathology, Toho University Omori Medical Center, Tokyo 143-8541;

²Department of Microbiology, Meiji Pharmaceutical University, Tokyo 204-8588;

³Laboratory of Human Nutrition, Wayo Women's University, Chiba 272-8533;

⁴Laboratory of Molecular Cell Biology, School of Pharmacy, Nihon University, Chiba 274-8555; and

⁵R&D Laboratories, Pola Pharma Inc., Yokohama 244-0812, Japan

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SUMMARY: The efficacy of polyene macrolides to treat experimental *Trichosporon* bloodstream infection was evaluated by histopathological examination and viable cell counts in the kidneys of infected mice. Viable cell counts on the 5th day after infection confirmed that liposomal amphotericin B (L-AMB) is a more effective treatment than fluconazole (FLC) for mice infected with an azole-resistant strain of *Trichosporon*. Histological examination revealed that the administration of L-AMB induced a transformation from acute purulent inflammation caused by both azole-susceptible and -resistant strain infections to a chronic and subsiding form, whereas FLC failed to convert the acute inflammation induced by the azole-resistant strain to a subsiding form. Our results demonstrate that polyene macrolides can be used as an alternative therapy for infection of azole-resistant strains of *Trichosporon* and that histopathological evaluation is useful for elucidating the pathophysiology of an experimental *Trichosporon* infection.

INTRODUCTION

Among dimorphic yeasts, *Trichosporon* spp. are the second most common cause of bloodstream infections following *Candida* spp. (1,2). The azole class of molecules is used as a first-line therapy for trichosporonosis (3), but recent studies have shown that some clinical isolations of *Trichosporon* spp. exhibit poor susceptibility to azole treatment (4–6). Therefore, in vivo evaluations of the efficacy of polyene macrolides against azole-resistant strains of *Trichosporon* spp. are being performed to determine whether this class of compounds can be used as alternative therapeutic agents. Typically, the assessment of the virulence of an organism and efficacy of therapeutic agents are determined by a survival curve or viable cell counts from organs of experimentally infected animals. Histopathological examination has rarely been adopted for such purposes. However, both these methods have the potential to examine interesting aspects of the pathophysiology of infection. Namely, histopathological examinations by light microscopy can evaluate the strength of fungal growth in tissue and assess the extent of infection and defense mechanisms of the host, which are typically indicated by several index findings including the distribution of lesions in organs, variety and strength of inflam-

matory infiltrates, tissue degeneration or necrosis, and deformity of invading fungi.

Therefore, we compared the efficacy of azoles and polyene macrolides by performing histological examinations and determine the viable cell counts in the kidneys of mice intravenously infected with azole-sensitive and -resistant *Trichosporon asahii* strains in order to determine the efficacy of polyene macrolides in treating trichosporonosis caused by azole-resistant strains and to assess the validity of histopathological examinations of the foci of an experimental infection.

MATERIALS AND METHODS

Fungi: *T. asahii* 336 (azole-resistant) and *T. asahii* 15 (azole-sensitive) maintained at Meiji Pharmaceutical University were used in this study. The MICs of these strains against representative antifungals are as follows: *T. asahii* 336: amphotericin B (AMB), 1 µg/ml; fluconazole (FLC), ≥64 µg/ml; voriconazole (VRC), 4 µg/ml; and *T. asahii* 15: AMB, 0.5 µg/ml; FLC, 8 µg/ml; VRC, 0.25 µg/ml.

Animals: In all experiments, 8-week-old Institute of Cancer Research male mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) weighing 30–39 g were used. All mice were housed in standard boxes with sawdust bedding and given food and water ad libitum. Five to seven mice were housed in each box. The experimental protocol was approved by the institutional review boards of Toho University for both animal and microbiological experiments (#DOSHO-11-21-159 and #BISHO-11-21-46, respectively).

Immunosuppression: Forty-eight and 24 h before

*Corresponding author: Mailing address: Department of Surgical Pathology, Toho University Omori Medical Center, 6-11-1 Omori-nishi, Ota-ku, Tokyo 143-8541, Japan. Tel: +81-3-3762-4151, Fax: +81-3-3767-1567, E-mail: yoichiro0207@hotmail.com

fungal inoculation, all mice received intraperitoneal injections of cyclophosphamide (Endoxan; Shionogi & Co., Osaka, Japan) at a dose of 200 mg/kg of body weight.

Infection: *T. asahii* was cultured on Sabouraud dextrose agar plates (SDA; Eiken Chemical Co., Tokyo, Japan) at 30°C for 48 h. Mature fungi were harvested using a disposable inoculating loop and suspended in 0.9% saline. The suspension was then filtered through a mesh filter (BD Falcon™ cell strainer; Nippon Becton Dickinson Co., Tokyo, Japan) to remove the hyphae. The suspension was composed of more than 90% of yeast. The suspension was then adjusted to 1×10^7 cells/ml and a 0.3-ml aliquot of the suspension (3×10^6 cells) was injected into the lateral tail vein of mice.

Administration of antifungals: Mice infected with the 2 different strains of *T. asahii* were randomly divided into 3 groups to receive either 5 mg/kg of liposomal AMB (L-AMB; Ambisome; Dainippon Sumitomo Pharma Co., Osaka, Japan) intravenously 24 and 48 h after infection, 40 mg/kg of FLC (Diflucan; Pfizer Japan, Inc., Tokyo, Japan) orally at 24 and 48 h after infection, or no treatment. Consequently, the following 6 experimental groups of mice were formed: azole-sensitive strain without treatment (SN), azole-sensitive strain inoculated with L-AMB (SL), azole-sensitive strain inoculated with FLC (SF), azole-resistant strain without treatment (RN), azole-resistant strain inoculated with L-AMB (RL), and azole-resistant strain inoculated with FLC (RF). Each group in the present study contained 11–16 mice (SN, $n = 14$; SL, $n = 11$; SF, $n = 12$; RN, $n = 16$; RL, $n = 11$; and RF, $n = 12$; 76 mice in total).

Mycological examination (viable cell counts): Kidneys were obtained from mice that were euthanized by cervical dislocation on the 5th day after infection. The right kidney of each mouse was bisected in the frontal section via the hilum renale. The ventral part of the kidney was homogenized in 1 ml of saline and subsequently diluted and plated onto SDA agar. Colonies were counted after incubation at 30°C for 48 h, and CFU per gram of body weight of each kidney was calculated.

Histopathological examination: The dorsal parts of the right and left kidneys were fixed in 10% buffered formalin, dehydrated with ethanol, and embedded in paraffin. Because the left kidney of each mouse was bisected in the frontal section via the hilum renal, the following 3 sections in each kidney were prepared for light microscopic observation (the dorsal part of the right kidney and the ventral and dorsal parts of the left kidney). Sections (3 μ m in thickness) were mounted on glass slides, stained with periodic acid-Schiff reaction, and examined using light microscopy. Three expert histopathologists identified the characteristics of lesions from each experimental group using representative parameters, including the total number and size of lesions, distribution pattern (cortex or medulla), the strength of yeast cell proliferation, and variety of inflammatory cells composing each lesion.

Data analysis: Differences in CFUs were determined by the Mann-Whitney *U* test. The correlation between CFUs and the number of lesions was also evaluated by the Spearman's rank correlation coefficient test. All statistical tests were considered significant at $P < 0.05$.

Fig. 1. CFUs in kidneys from experimental groups. The SN and RN groups showed significantly higher viable cell counts than other groups ($P < 0.01$) that received antifungal treatment. The RF group showed significantly higher viable cell counts than the SF group ($P < 0.05$). There was no significant difference between any other pair of groups. SN, azole-sensitive strain without any treatment; SL, azole-sensitive strain inoculated with L-AMB; SF, azole-sensitive strain inoculated with FLC; RN, azole-resistant strain without any treatment; RL, azole-resistant strain inoculated with L-AMB; and RF, azole-resistant strain inoculated with FLC. Each value is expressed as the mean and standard deviation. Differences were considered to be significant at $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$).

RESULTS

Viable cell counts in kidneys: The average viable cell counts for kidneys from the SN, SL, SF, RN, RL, and RF groups were 1.39×10^7 , 4.06×10^5 , 2.22×10^5 , 7.60×10^6 , 4.58×10^5 , and 7.56×10^5 CFU/g, respectively (Fig. 1). Despite the differences concerning the type of infected strain and antifungals administered, mice treated with an antifungal showed significantly lower viable cell counts than those that were not treated ($P < 0.01$). However, the RF group showed significantly higher viable cell counts than that observed in the SF group ($P < 0.05$).

Histopathological examinations: (i) Macroscopic findings: The external surface and section of kidneys from the SN and RN groups exhibited approximately 20 foci of diffusely spread, yellowish-white granuli less than 1 mm in diameter. In contrast, kidneys from the 4 treated groups showed none or few granuli.

(ii) Microscopic findings: Sections of kidneys from the SN group showed scattered foci (Fig. 2a). Large (more than 500 μ m in diameter) and round-shaped lesions were predominantly present in the cortical area, the center of which was composed of densely clustered yeast with eccentric hyphal growth aligned in a radial pattern, which was encompassed by dense inflammatory infiltrate largely composed of segmented leukocytes (Fig. 2b). On the other hand, most lesions in the medulla were elongated and oval-shaped, consisting of clustered yeast and segmented leukocytes in the lumen of the distal tubule. These tubules were encompassed by dense inflammatory infiltrates, mainly composed of segmented leukocytes (Fig. 2c). Less common were mononuclear leukocytes recruited in any of the foci of this group.

The number of lesions observed in the SL group was lesser than that in the SN group, and larger foci were

Fig. 2. Histopathology (periodic acid-Schiff stain) of kidneys with azole-sensitive *Trichosporon asahii* infection. (a) Some large and round-shaped lesions were present predominantly in the cortical area in the kidney of the SN group. (b) A typical lesion in the cortex showed many yeasts densely clustered with hyphal growth in a radial pattern and enclosed by dense segmented leukocyte infiltrates. (c) Lesions in the medulla were elongated and oval-shaped, which consisted of clustered yeast and included segmented leukocytes filling the lumen of the distal tubule. (d, e, f, and g) The kidneys of the SL and SF groups presented fewer and smaller lesions, which showed weaker fungal proliferation than those of the SN group. a, SN, 40 $\times$; b, SN, cortex, 400 $\times$; c, SN, medulla, 400 $\times$; d, SL, 40 $\times$; e, SL, 400 $\times$; f, SF, 40 $\times$; and g, SF, 400 $\times$.

rarely observed (Fig. 2d). Most foci were small- to medium-sized (less than 500 μm in diameter) and oval in shape, and yeast were sparsely present and limited to the cluster of inflammatory cells comprising segmented and mononuclear leukocytes (Fig. 2e). Mononuclear cells occasionally have PAS-positive fine granuli in their cytoplasm, which may correspond to a phagocytized fungal component. The histological features of the lesions developed in the SF group were essentially the same as those observed in the SL group (Figs. 2f and 2g).

In the RN group, many round-shaped foci were scattered over the kidneys, for which histological findings such as the size of foci, features of fungal growth, and

type of inflammatory infiltrate were mostly the same as those in the SN group (Figs. 3a, 3b, and 3c).

The number of lesions detected in the kidneys was the lowest in the RL group, which were mostly less than 200 μm in diameter and consisted of densely clustered mononuclear cells. Additionally, yeasts were rarely observed in foci (Figs. 3d and 3e). The RF group exhibited an intermediate number of lesions, a large portion of which were medium-sized (200–500 μm in diameter) and comprised a dense cluster of segmented leukocytes (Fig. 3f). Larger lesions as a minor member of this group contained yeasts with sporadic hyphal growth (Fig. 3g).

To determine the relationship between CFUs and the histological characteristics of lesions in kidneys, major

Fig. 3. Histopathology (periodic acid-Schiff stain) of kidneys with azole-resistant *Trichosporon asahii* infection. (a, b, and c) Many round-shaped foci were scattered over kidneys of the RN group, and histological findings such as the size of foci, features of fungal growth, and the type of inflammatory infiltrate were mostly the same as those of the SN group. (d and e) In kidneys from the RL group, there were an extremely small number of lesions without yeasts that were smaller than 200 μm in diameter. (f and g) The RF group showed an intermediate number of lesions, which was composed of a dense cluster of segmented leukocytes. a, RN, 40 $\times$; b, RN, cortex, 400 $\times$; c, RN, medulla, 400 $\times$; d, RL, 40 $\times$; e, RL, 400 $\times$; f, RF, 40 $\times$; and g, RF, 400 $\times$.

histopathological findings with regard to representative parameters were observed in the present study. The following parameters exhibited a close relationship to CFUs recovered from the kidneys of infected mice: high numbers of large lesions (Fig. 4), yeasts with extensive hyphal growth (Fig. 5), and segmented leukocytes (Fig. 6). In contrast, there was no difference between the ratio of lesions found in the cortex and those found in the medulla (Fig. 7).

In addition, a significant relationship between CFUs and the number of lesions emerged after the rank correlation coefficient was evaluated. This correlation was further emphasized by selecting lesions that mostly consisted of segmented leukocytes, which are major histological indicators of the degree of acute purulent inflam-

mation (Figs. 8a and 8b) (7).

DISCUSSION

Several cases of invasive trichosporonosis have been reported (8,9) since its first report in 1970 (10). In 2004, a case series reported that the overall prevalence of trichosporonosis was 8 per 100,000 admitted patients, with 65% of infected patients having acute leukemia and 65% exhibiting neutropenia. The mortality rate at 30 days after admission was 53% (11). Today, *Trichosporon* bloodstream infection is known as one of the most serious fungal infections in immunocompromised patients. The use of the azole class of compounds is generally accepted as a first-line therapy for

Fig. 4. Number and population of 3 different-sized lesions. The number of different-sized lesions of each group. SN and RN groups presented more than 20 lesions and a high proportion of large-sized lesions. With the exception of the RF group, the groups that received the antifungal treatment presented a fewer number of lesions. The ratio of large-sized lesions was essentially in proportion to the total number of lesions. Black, more than 500 μm in diameter; dark shaded, 200–500 μm in diameter; and faint shaded, less than 200 μm in diameter.

Fig. 5. Representative feature of lesions indicating the number of fungi. (a) The percentage of lesions categorized based on the strength of fungal growth. In the SN and RN groups, lesions that fell into grade 2 or 3 accounted for approximately 50% of all lesions. The RF group was next to these non-treatment groups in regard to proliferation of fungi. The majority of lesions in the other 3 groups belonged to grade 0 or 1. Black, grade 3; dark shaded, grade 2; faint shaded, grade 1; and white, grade 0. (b) Explanation of the 4 scores from 0 to 3. Grade 0, none; grade 1, very few fungi present sparsely and limited in a cluster of inflammatory cell infiltrate; grade 2, several yeast cells and/or hyphae present at medium density; grade 3, yeast cells clustered densely in the center of a lesion and expand in hyphal form toward the peripheral area.

Fig. 6. Ratio of lesions largely composed of inflammatory cells. The percentage of lesions typed by inflammatory cell infiltrates. Considering the number of lesions with mostly segmented neutrophil infiltrates, the following represent the ratio of these lesions from groups in the descending order: non-treatment group, FLC treatment group, and L-AMB treatment group. In particular, the RL group mostly consisted of lesions with mononuclear cell infiltrates. Black, predominantly segmented leukocytes; dark shaded, relatively similar amounts of segmented leukocytes and mononuclear cells; faint shaded, predominant mononuclear cells; and white, very few inflammatory cell infiltrates.

Fig. 7. Distribution: cortex/medulla. The percentage of lesions present in the cortex and medulla. With the exception of mice with very few lesions, the ratio of cortex to medulla was approximately 2.5 to 1. A constant correlation was observed regardless of the strains and treatments used. Black, cortex; and dark shaded, medulla.

this disease (3), but azole-resistant strains have been reported (12). Although echinocandins exhibit poor efficacy for the disease (13), polyene macrolides can be considered candidates for the treatment. However, the efficacy of polyene macrolides for the treatment of experimental infection of azole-resistant strains needs to be elucidated to provide appropriate support for their clinical use. In this study, we compared the efficacies of polyene macrolides and azoles using a mouse model of *Trichosporon* bloodstream infection with azole-sensitive and -resistant strains. In addition, the usefulness of histopathological examination was also evaluated to determine details of the pathophysiology of the experimental infection. We used FLC among azoles because this molecule has been generally used for both

Fig. 8. Correlation between CFUs and the number of lesions. (a) Correlation between CFUs and the number of whole lesions. A moderate positive correlation was found between CFUs and the total number of lesions ($P < 0.001$, Spearman's rank correlation coefficient = 0.631). (b) Correlation between CFUs and the number of lesions with segmented leukocyte infiltrates. A high positive correlation was determined between CFUs and the number of lesions with acute purulent inflammation ($P < 0.001$, Spearman's rank correlation coefficient = 0.670). The results were obtained from all mice regardless of the type of infected strain or treatment.

treatment and prophylaxis. Additionally, we tried to employ better methods of administration and considered the appropriate pharmacokinetic/pharmacodynamic parameters of each antifungal agent for potential clinical usage (14,15). Administration of both antifungal agents significantly decreased viable cell counts regardless of the differences between the infected strains. This result is in agreement with those of a previous study on experimental trichosporonosis (16). Importantly, L-AMB treatment was effective against both strains, whereas FLC exhibited a poorer efficacy for mice infected with the azole-resistant strain. These findings confirm the usefulness of polyene macrolides for the treatment of *Trichosporon* bloodstream infection caused by azole-resistant or poorly susceptible strains.

The histopathological diagnosis of infectious diseases, especially for fungal infections, has played an important role in providing appropriate information for clinical management, which determines not only defini-

tive and suspicious causative fungi but also the extent and nature of impaired defense mechanisms of the host. However, this method has rarely been applied for the evaluation of an experimental infection, which may likely be due to subjectivity. We verified the reliability and usefulness of histopathological examinations in evaluating the extent of an experimental infection. Histological features of lesions facilitated their classification into 3 patterns based on differences in the strength of fungal proliferation and type of inflammatory infiltrate. The 2 non-treated groups (SN and RN) infected with different strains showed many large lesions consisting of extensive fungal proliferation with significant segmented leukocyte infiltrates, whereas 3 of the treated groups (SL, SF, and RL) showed a few small lesions in the cortical area that were composed of mononuclear cells and a small number of fungal cells. RF was the only group that showed a transitional form among the other groups. Dense infiltrates of segmented leukocytes and mononuclear cells suggest the existence of acute and chronic inflammation, respectively (7). An effective antifungal could induce a change from an acute and purulent inflammation to a chronic form by suppressing fungal proliferation in vivo. Histological evaluation of the strength of fungal proliferation and segmented leukocyte infiltrates revealed a significant relationship with CFUs from infected kidneys. In addition, we observed a predominant distribution of uniform purulent foci in the cortical area, which was recognized as an abscess and confirmed that the pathophysiology was similar to that of human disease developed in rodents (17). The reproducibility of the model was also confirmed by histological observations. Accordingly, histopathological evaluation can be a useful supplemental methodology to determine the pathophysiology of an experimental infection as this evaluation provided more detailed information about differences in host responses to fungal infections.

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